BIOMEDICAL LIBRARY
OPEN SHELF
CURRENT

THE

AUSTRALIAN JOURNAL

OF

EXPERIMENTAL BIOLOGY

AND

MEDICAL SCIENCE

1960

EDITOR-IN-CHIEF
MARK L. MITCHELL

CO-EDITOR
G. M. BADGER

VOL. XXXVII

PUBLISHED BY THE UNIVERSITY OF ADELAIDE

PRINTED AT THE GRIFFIN PRESS, MARLBOROUGH PLACE, ADELAIDE
1959

B570.5
Au78

CONTENTS

PART 1. FEBRUARY, 1959

PAGE

P. Shepherd and W. J. Simmonds: Some conditions affecting the maintenance of a steady lymphatic absorption of fat - - - - - - 1

A. K. Lascelles and P. Manusu: The healing of surgical wounds in the skin of the sheep - - - - - - - - - - - - - 11

Marie L. Emanuel: The susceptibility of mice to North Queensland strains of leptospirae - - - - - - - - - - - 17

Ilse Brading, Shirley A. Cantrill and R. J. Walsh: Removal of haemoglobin from the blood of normal and nephrectomised rats - - - 31

Ilse Brading, E. P. George and R. J. Walsh: The effect of receptor destroying enzyme, trypsin and papain on red cell survival - - - 37

A. M. Snoswell: Flavins of *Lactobacillus arabinosus 17.5*. A lactic dehydrogenase containing a flavin prosthetic group - - - - - 49

R. K. F. Pak Poy and J. S. Robertson: An electron microscopical study of glomerular changes in experimental hydronephrosis in rats - - 65

E. R. Trethewie and C. Rumberg: The effect of salicylate on the *in vitro* antigen-antibody reaction in anaphylaxis - - - - - - - 77

P. C. Vincent: The effects of heavy metal ions on the human erythrocyte. 3. Inhibition of the effects of lead and mercury - - - - - - 83

V. Elizabeth Hodgetts: The influence of centrifugational treatment upon haematocrit values and the trapped plasma correction factor of sheep blood. - - - - - - - - - - - - - - 97

Henry Francis Holden: A simple reservoir burette for alkaline solutions - 107

U OF M BINDERY SEP • 59 (B)

PART 2. APRIL, 1959

PAGE

W. G. NAYLER: The effect of strophanthin-G on the rate of deamination of adenyl compounds - - - - - - - - - - - - 109

J. H. POPE: A virus of the encephalomyocarditis group from a water-rat, *Hydromys chrysogaster*, in North Queensland - - - - - - 117

G. WOOLLEY AND W. J. SIMMONDS: The effect of operation and chronic lymph fistula on production of tissue fluid and lymph - - - - 125

JUNE B. SHEATH: Factors in the colorimetric estimation of 17-ketosteroids in urine - - - - - - - - - - - - - - 133

JUNE B. SHEATH: Chromatography of urinary ketosteroids - - - - 147

R. K. F. PAK POY: Electron microscopy of the reptilian renal glomerulus - 153

PATRICIA E. LIND: Further studies of an avirulent mutant of the influenza A strain WS— - - - - - - - - - - - 163

HENRY FRANCIS HOLDEN: The determination of urea in blood - - - - 177

DOLORES HOLT: Presence of K-virus in wild mice in Australia - - - - 183

PART 3. JUNE, 1959

PAGE

G. N. Cooper: Experimental Shigellosis in mice. I. Chronic infection with *Shigella dysenteriae* type 2 - - - - - - - - - 193

G. N. Cooper and Jill A. Pillow: Experimental Shigellosis in mice. II. Immunological responses to *Shigella dysenteriae* type 2 infection 201

J. R. Goding and D. A. Denton: The response to Na^+ depletion in adrenalectomized sheep with parotid fistulae - - - - - - 211

J. Marcolis: Hageman factor and capillary permeability - - - - - 239

Janet G. Campbell: Anamnestic responses in rabbits immunized with two related bacterial viruses - - - - - - - - - - - - 245

Ian Cook and J. H. Hope: *Toxoplasma* in Queensland. III. A preliminary survey of animal hosts - - - - - - - - - - - - 253

R. G. Webster: The isolation of orphan viruses from pigs in New Zealand 263

H. D. Purves and Nancy E. Siretт: A study of the hormone contents of the rostral and caudal zones of the pars anterior of the wallaby pituitary - - - - - - - - - - - - - - - - 271

Winifred G. Nayler: The action of fluothane, chloroform and hypothermia on the heart - - - - - - - - - - - - - 279

E. P. George and W. Sollich: The adsorption of I^{131} iodide ions on plasma proteins - - - - - - - - - - - - 289

A. J. Day, A. R. Johnson, M. W. O'Halloran and C. J. Schwartz: The effect of the antioxidant butylated hydroxy toluene on serum lipid and glycoprotein levels in the rat - - - - - - - - - 295

J. E. O'Hagan: An efficient, inexpensive dialyser - - - - - - 307

PART 4. AUGUST, 1959

PAGE

P. FANTL: The influence of certain metal salts on blood coagulation - 309

C. C. KRATZING AND G. M. WINDRUM: The effect of ethyl trichloracetate on liver lipids - - - - - - - - - - - - - - 321

D. R. LAMOND: Ovarian transplants in ovariectomised mice - - - 331

E. A. NORTH: The use of specific antitoxin against experimental staphylococcal infection in mice - - - - - - - - - - - 341

S. G. ANDERSON AND G. L. ADA: Murray Valley encephalitis virus: preparation of an infective "ribonucleic acid" fraction - - - - - - 353

R. L. DOHERTY, J. G. CARLEY AND PATRICIA E. LEE: Studies of arthropod-borne virus infections in Queensland. I. A serological survey of aboriginal missions bordering the Gulf of Carpentaria - - - - 365

T. R. BRADLEY AND J. F. NELSON: The effect of alloxan diabetes on the metabolic activity of rat mammary gland tissue - - - - - - 373

GORDON T. ARCHER: The release of histamine from the mast cells of the rat 383

K. D. G. EDWARDS: Measurement of antipyrine in plasma: interference by certain drugs - - - - - - - - - - - - - - 391

A. W. TURNER AND V. ELIZABETH HODGETTS: The dynamic red cell storage function of the spleen in sheep. I. Relationship to fluctuations of jugular haematocrit - - - - - - - - - - - - - 399

C. H. GALLAGHER AND L. E. A. SYMONS: Biochemical studies on *Nippostrongylus muris* infestation - - - - - - - - - - 421

PART 5. OCTOBER, 1959

PAGE

R. G. WALES: The differential staining of human and dog spermatozoa - 433

I. G. WHITE: Studies on the estimation of glycerol, fructose and lactic acid with particular reference to semen - - - - - - - 441

F. C. COURTICE: Permeability of normal and injured skin capillaries to lipoproteins in the rabbit - - - - - - - - - - - 451

F. C. COURTICE: The peameability of liver and skin capillaries to lipids in the cat - - - - - - - - - - - - - - 465

JOHN S. CHARNOCK, BRIAN F. GOOD, AND BASIL S. HETZEL: The effect of salicylate on plasma protein bound iodine in man - - - - - 473

J. R. CASLEY-SMITH: The haematology of the Central Australian aborigine. 11. White and differential counts; eosinophil counts and Casoni tests 481

E. E. SELECKI: A study of the metastatic distribution of Erlich ascites tumour cells in mice - - - - - - - - - - - - 489

G. J. V. NOSSAL: Plasma cell proliferation following whole body irradiation 499

MAUREEN F. LARKIN: The establishment of a strain of human cells in tissue culture - - - - - - - - - - - - - - 505

PART 6. DECEMBER, 1959

PAGE

JOHN S. CHARNOCK, JOHN CASLEY-SMITH AND COLIN J. SCHWARTZ: Serum magnesium-cholesterol relationships in the Central Australian aborigine and in Europeans with and without ischaemic heart disease - 509

J. R. CASLEY-SMITH: The haematology of the Central Australian aborigine. III. Lymphocyte and neutrophil haemograms, with European controls 517

W. D. BROWN: Determination of serum cholesterol with perchloric acid 523

W. D. BROWN, A. R. JOHNSON AND M. W. O'HALLORAN: The effect of the level of dietary fat on the toxicity of phenolic antioxidants - - 533

ANNE G. JABARA: Canine ovarian tumours following stilboestrol administration - - - - - - - - - - - - - - 549

E. R. TRETHEWIE AND C. W. MORRIS: The inhibitory effect of salicylate on the release of histamine by venom in the presence of blood - - 567

E. R. TRETHEWIE: The effect of cortisone on the release of gastric acid by histamine - - - - - - - - - - - - - 573

IAN COOK: Toxoplasma in Queensland. IV. Serological surveys of the human population - - - - - - - - - - - - 581

I. K. BUCKLEY AND J. SEMKIW: A high frequency pulse generator for injuring tissue within the Sandison-Clark rabbit ear chamber - - - 593

SOME CONDITIONS AFFECTING THE MAINTENANCE OF A STEADY LYMPHATIC ABSORPTION OF FAT

by P. SHEPHERD AND W. J. SIMMONDS[1]

(From the Physiology Department, University of Western Australia).

(*Accepted for publication 10th October, 1958.*)

SUMMARY.

The hourly output of fat, the rate of flow and the concentration of fat in the thoracic duct lymph were measured during 8 hours steady infusions of 125, 250 and 500 μ Eq of esterified fat per hour into the duodenum of unanaesthetized rats.

At the two lower rates of infusion, a steady state of transfer of fat from the intestinal lumen to the lymph was obtained after 4 hours, and the rate of lymph flow and the concentration of fat in the lymph were steady after the 5th hour. At the highest infusion a steady state was not obtained, suggesting a limit to the rate at which fat can be transferred to the lymph.

The effect of extra fluid administration on the steady state of transfer with infusions of 250 μ Eq of fat per hour was examined over 10 hours. Fat output was significantly enhanced when the additional fluid was given by duodenum. When fluid was given by jugular vein the rate of lymph flow was increased, but the hourly fat output was not significantly affected. A possible mechanism for the effect of fluid absorption on fat output is discussed.

INTRODUCTION.

Previous work on the lymphatic absorption of fat has dealt mainly with single doses of fat administered intragastrically. While this technique has provided valuable data, the absorption of fat is affected by the rate of stomach emptying and the conditions for absorption are continually changing. Hence it seemed of interest to establish, if possible, a steady state of transfer of fat from the lumen of the intestine to the lymph. This would facilitate a quantitative study of factors concerned in the absorption of fat. This paper presents experiments which show that such a steady state can be maintained by intraduodenal infusion of fat emulsion and that there is a limit to the rate at which a steady transfer of fat to the lymph can be obtained. It also seems that, to a limited extent, the process is enhanced by the rapid absorption of fluid concurrently with fat.

---

[1] This work was carried out with the aid of a grant from the National Health and Medical Research Council, Canberra.

METHODS.

Adult male and female rats weighing 190 to 230 gm. were used. A "Transflex" tubing cannula was inserted into the abdominal thoracic duct (Bollman, Cain and Grindlay, 1948) under ether anaesthesia. A very flexible "Transflex" polyvinyl tube (softened with Dioctyl phthallate) was inserted into the stomach, threaded through the pylorus and passed into the duodenum, so that the tip of the tube was lying about 6 cm. distal to the pyloric sphincter. This tube was stitched into position in the stomach wall and also sutured firmly at the body wall. In the animals in which fluid was administered intravenously, a plastic tube filled with heparinized saline was inserted into the right jugular vein and taken through a stab wound at the back of the neck. After sewing all three tubes into position, the wounds were sutured and the animal placed in a restraining cage (Bollman, 1948).

Immediately following the operation, the duodenal tube was connected to a constant speed infusion apparatus and 1·2 ml. of Ringer-Locke per hour were administered for 5-12 hours. The rate of fluid administration overnight was varied according to the experimental treatment planned for the following day. Prior to experiments where 0·55 ml. of emulsion was given per hour, Ringer-Locke was given at 0·6 ml./hr., while before experiments where 1·1 ml. of emulsion was given per hour, 1·2 ml. of Ringer-Locke was administered per hour. The collection of lymph, care of fistulae and housing of animals were as described previously (Simmonds, 1954).

Experimental procedure.

Experiments were commenced at either 8 a.m. or 9 a.m. and continued for 8 or 10 hours. Lymph was collected at hourly intervals during this experimental period while the overnight collection (14 to 16 hr.) was pooled. During the experimental period, fat emulsion was fed at a constant speed into the duodenal tube, and in one group of experiments Ringer-Locke was simultaneously administered at a constant speed *via* the venous cannula. The fat emulsion was "Nucrose", an oral fat emulsion containing 50 p.c. coconut oil. Dilutions of stock emulsion were made up volume by volume in 0·9 p.c. sodium chloride, immediately before use.

Prior to the commencement of fat infusion, a "priming" dose of diluted emulsion was given to fill the dead space in the duodenal cannula and to shorten the time interval required for fat to be spread over the intestinal surface. In all cases, this dose was injected *via* the duodenal tube and contained 0·05 ml. of coconut oil in 0·3 ml. of fluid.

Analytical methods. The total esterified fat in the lymph was measured by the method of Stern and Shapiro (1953).

Fluids administered.

The composition of the Ringer-Locke given to the animals was as follows:—

Sodium chloride, 9·00 gm.; Potassium chloride, 0·42 gm.; Calcium chloride, 0·24 gm.; Sodium bicarbonate, 0·20 gm.; Glucose, 1·00 gm.; Distilled water to 1 litre.

Repeated analysis of the "Nucrose" for total esterified fat by the method of Stern and Shapiro gave a mean value of 2270 μ Eq esterified fat/ml. of "Nucrose" (≡ 0·5 ml. of coconut oil).

RESULTS.

Six groups of experiments were performed. In groups 1, 2 and 3 the effects of increasing the rate of fat administration were determined. In groups 4, 5 and 6 the effect of extra fluid given intraduodenally (groups 4 and 5) or intravenously (group 6) on fat output in the lymph was tested.

Increasing rate of fat administration.

In groups 1, 2 and 3 the rates of fat administration were respectively 125, 250 and 500 μ Eq/hr., but the rate of fluid administration was the same for all, being 0·55 ml./hr. Fig. 1 shows the average fat outputs in lymph and Fig. 2 the average rates of lymph flow and concentrations of fat in lymph during 8 hr. infusion. In groups 1 and 2 a steady state was achieved (see A and B, Figs. 1 and 2). Fat output became steady first, by 3rd to 4th hour of infusion, while lymph flows and fat concentrations varied little after the 5th hour. Doubling the rate of fat administration, 125 to 250 μ Eq/hr., produced a comparable increase in the steady rate of fat output in lymph, 75 to 125 μ Eq/hr. The average output of fat in the lymph of fasting animals in these and in previous experiments was about 20 μ Eq/hr. If this 'endogenous' fat output were subtracted, the output of absorbed administered fat would have been 55 and 105 μ Eq/hr. respectively. Over the dose range used for groups 1 and 2, then, the rate of fat absorption was clearly proportional to the rate of administration.

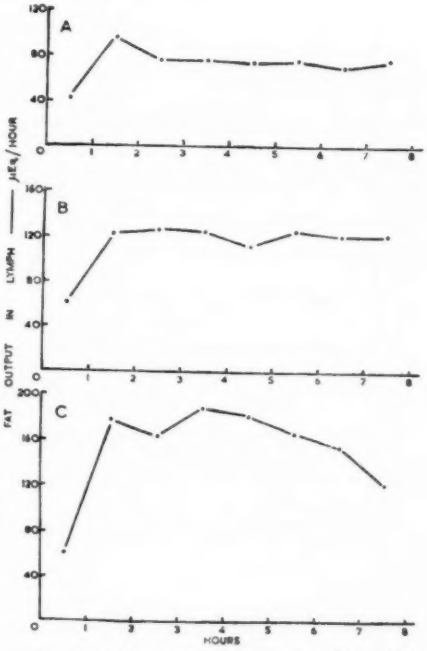


Fig. 1. Mean output of fat in μ Eq esterified fat per hour during an 8 hour steady infusion of fat. A—125 μ Eq fat/hour; B—250 μ Eq. fat/hour; C—500 μ Eq fat/hour.

In group 3, a steady state was not achieved (Figs. 1C and 2C). The mean output of fat in the lymph reached 188 μ Eq in the 4th hour, but showed a steady decrease thereafter. During the last 4 hours, the output of fat showed a mean decrease of 16 μ Eq of fat per hour, this decrease being statistically significant at the 5 p.c. level.

Increased fluid loading.

In group 4, consisting of 4 experiments in 3 rats, fat was given at the same high rate as in group 3, 500 μ Eq/hr., but with twice the volume of fluid – 1·1 ml./hr., before and during infusions of fat. Lymph flows were higher and fat concentrations in lymph were lower in group 4 but fat outputs were much the same as in group 3, and a steady state was not achieved during 8 hour infusion.

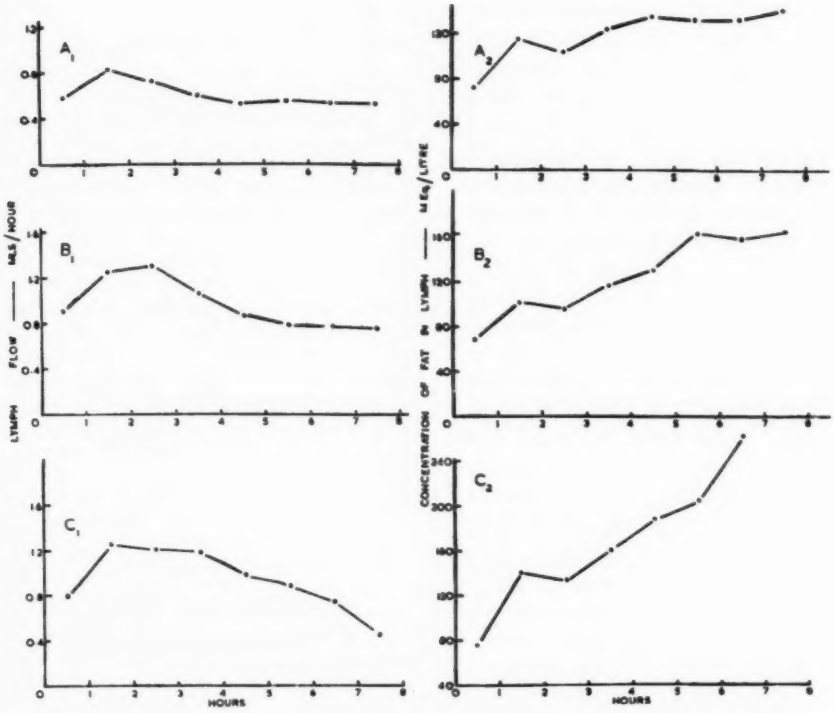


Fig. 2. Left-hand side. Mean lymph flow in ml. per hour during an 8 hour steady infusion of fat. A_1—125 μ Eq fat/hour; B_1—250 μ Eq fat/hour; C_1—500 μ Eq fat/hour.
Right-hand side. Mean concentration of fat in lymph in m. Eq esterified fat per litre during an 8 hour steady infusion of fat. A_2—125 μ Eq fat/hour; B_2—250 μ Eq fat/hour; C_2—500 μ Eq fat/hour.

The results obtained were more variable than those obtained in other groups. In all other groups there was no statistically significant variation between animals, while this group showed a highly significant variation ($P < \cdot 001$).

Group 5 consisted of five, 10-hour experiments on 4 animals. For the first 4 hours of the experimental period, the animals were treated identically with those in group 2. That is, during this period 250 μ Eq of esterified fat were given in 0·55 ml. of fluid during each hour. At the end of 4 hours the rate of instillation of fluid was doubled, but the dose of emulsified fat remained the same. From the 5th to the 10th hour, 250 μ Eq of esterified fat in 1·1 ml. of fluid were given per hour.

The mean results for the first 4 hours were not significantly different from

the results obtained in group 2. The fat output had reached a steady value of about 130 μ Eq/hr. when the rate of fluid administration was doubled. Immediately following the change of treatment the output of fat in the lymph increased, the output of fat during the next 4 hours for this group being significantly greater than that for group 2 during the corresponding period. Also, the output achieved a steady level, about 190 μ Eq esterified fat per hour in hours 8, 9 and 10, that is the 4th, 5th and 6th hour after the change of treatment (Fig. 3).

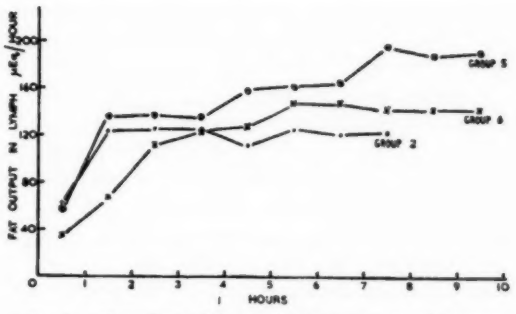


Fig. 3. The effect of fluid administration on the mean output of fat in μ Eq esterified fat/hour.

●——● animals received 250 μ Eq fat/hour in 0·55 ml. of fluid (group 2).

○——○ animals received 250 μ Eq fat/hour. Fat in 0·55 ml. of fluid during hours 1-4 and in 1·1 ml. of fluid in hours 5-10 (group 5).

×——× animals received 250 μ Eq fat/hour in 0·55 ml. of fluid by duodenum and simultaneously 1·73 ml. of fluid by jugular vein (group 6).

Lymph flow also became steady at a rate greater than in group 2, while fat concentrations were steady by the 7th hour (Fig. 4).

Group 6. This was a group of five 10-hour experiments performed on 4 rats which received the same dose of fat and fluid by intestine as in group 2. Throughout the entire experimental period, however, Ringer-Locke was infused into the jugular vein at a rate of 1·73 ml./hr. A steady state of fat output was achieved during the 6th hour at a level of 140 μ Eq of esterified fat per hour, while lymph flow and concentration reached a steady state in the 7th hour.

The steady hourly output of fat was not significantly different from that in group 2, but was significantly less than in group 5. Thus, when the rate of fat administration was kept the same in groups 2, 5 and 6, both fat output and lymph flow were enhanced during more rapid intestinal absorption of fluid. But with intravenous saline, lymph flow was increased without significant effect on fat output in the lymph (Figs. 3 and 4).

Percentage recovery of administered fat.

Two or three experiments were usually performed on each rat, different types of experiment being carried out on successive days. At least 14 hours elapsed between the cessation of one fat infusion and the commencement of the next. By this time the lymphatic absorption of fat appeared to be complete since sample measurements of fat concentration made during the 14th hour

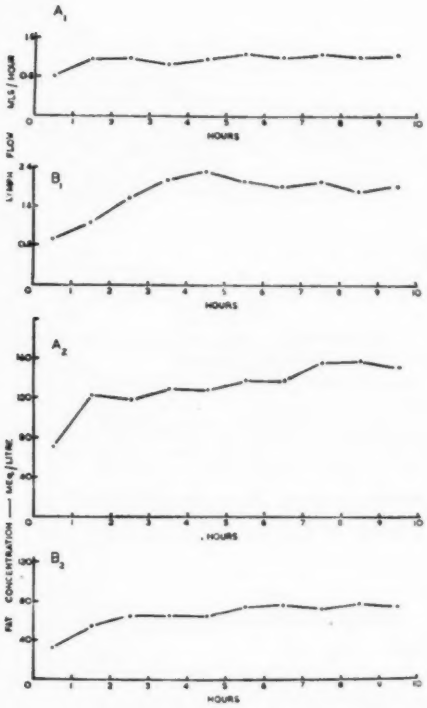


Fig. 4.

A_1 The lymph flow in ml./hour over a 10 hour period for animals receiving 250 μ Eq fat/hour in 0·55 ml. of fluid for first 4 hours, and in 1·1 ml. of fluid for last 6 hours (group 5).

B_1 The mean lymph flow in ml./hour over a 10 hour infusion period for animals receiving 250 μ Eq fat in 0·55 ml. plus fluid by jugular vein (group 6).

A_2 Mean fat concentration in the lymph in m. Eq per litre for animals in group 5.

B_2 Mean fat concentration in lymph in m. Eq per litre for animals in group 6.

approximated fasting values. The total fat collected during the infusion period, and the total for the 24 hour period, were corrected by subtraction of the mean fasting output of 20 μ Eq esterified fat per hour. These "net" figures were used to calculate the percentage recovery of the administered dose. The averages of such estimates for each group are given in Table 1.

TABLE 1.

Percentage recoveries of administered fat.

Group No.	Total dose	Percentage recovery during infusion period	Percentage recovery during 24 hours
1 (4)*	1226	35	53
2 (5)	2225	34	54
3 (4)	4223	25	52
4 (4)	4223	27	58
5 (5)	2725	49	63
6 (5)	2725	36	51

* Figure in brackets indicates the number of experiments in the group.

Comparing percentage recoveries during the infusion period for the three rates of fat infusion, these were the same at the two lower dose levels, groups 1 and 2, with which a steady state was obtained. The highest dose, with which a steady state was not obtained, produced a significantly lower percentage recovery during infusion ($P < 0{\cdot}01$). There was no significant difference between the two groups, 3 and 4, which received the same high dose of fat given in different volumes of fluid. However, with a fat dosage at which a steady state of absorption could be maintained, groups 2, 5 and 6, additional intestinal fluid in group 5 significantly enhanced the percentage recovery during the infusion period ($P < 0{\cdot}01$) but additional fluid by vein, group 6, did not. Irrespective of any differences during the infusion period, the percentage recoveries were much the same during the full 24 hr. from commencement of one infusion to beginning of the next experiment, there being no significant differences between the 6 groups. This uniformity together with the low hourly fat outputs at the end of the period, similar to those in fasting animals, supported the belief that absorption of the dose given in an experiment was completed before the next experiment began.

DISCUSSION.

From these experiments it appeared that a steady state of fat absorption could be maintained with infusions into the duodenum at rates up to 49·5 mg. of coconut oil per hour (0·55 ml. of 1:5 dilution of "Nucrose" emulsion per hour), but not with double that dose. Since the average weight of the animals used was 205 gm. the limiting dose lay between 24 and 48 gm./100 gm. body weight/hr., or between 16 and 32 mg./100 cm^2 body surface/hr. The rate at which absorbed fat appeared in the lymph was considerably less, accounting in each hour of the steady state period for approximately 50 p.c. of the esterified fat administered. It seems likely, however, that the true rate of absorption from the intestinal lumen was greater than the lymphatic recovery. Up to the limit mentioned above intestinal absorption probably matched the steady rate of administration quite closely.

A number of factors could contribute to this discrepancy between absorption rate from the lumen and the apparent recovery rate in the lymph, the most important being failure to collect all the absorbed fat through the fistula. Some intestinal lymph may not pass into the thoracic duct fistula. However, the high recoveries of labelled long-chain triglycerides (Bloom, Chaikoff, Reinhardt, Entenman and Dauben, 1950; Bloom, Chaikoff and Reinhardt, 1951; Borgström, 1951; Chaikoff, Bloom, Stevens, Reinhardt and Dauben, 1951) suggest that only a small portion normally escapes collection. More significant would be the diversion of lower molecular weight fats into the portal blood stream after absorption (Kiyasu, Bloom and Chaikoff, 1952). Indeed, butyric acid and tributyrin are not recovered in the lymph at all (Hughes and Wimmer, 1935; Simmonds, 1955).

Coconut oil contains a fairly high proportion of short-chain fats (Hilditch, 1947) and this could perhaps account for the discrepancy between absorption of this oil and that of olive oil which consists mainly of long-chain triglycerides. In the 24-hour recoveries measured in the present experiments, only about 50 p.c. of administered dose was obtained, whereas previous recoveries of olive oil yielded 70 to 80 p.c. Digestibility experiments show that the absorption from the intestine of both olive and coconut oils is virtually complete, about 90 p.c. of administered oil being removed from the intestinal lumen (Hoagland and Snider, 1943a, b). In our experiments, only the esterified fat in the lymph was measured, but normally the percentage of free fatty acids is not high.

Deuel, Hallman and Leonard (1940) studied in considerable detail rates of absorption from the intestinal lumen for many types of fat. These studies were made after single doses of fat had been given by gastric intubation. Their results suggest that there is an upper limit to the rate at which steady absorption from the lumen is maintained. Considerable differences between the techniques used by Deuel *et al.* and those used for the present series of experiments preclude close comparison of the results obtained in the two cases. However, it does seem that their limiting value was 2-3 times the highest infusion rate at which a steady lymphatic output was maintained in our rats.

A detailed study of the factors which may limit a steady transfer of fat from the intestinal lumen to the lymphatics has not been made as yet. Increased lymph flow after the intravenous saline did not substantially increase the fat output in the lymph. Hence, it appeared that the flow of tissue fluid with absorbed fat into lymphatics was normally sufficient to balance the addition of fat to tissue fluid by the absorbing epithelial cells. However, an increased flow of fluid absorbed from the lumen through epithelial cells appeared to carry with it some extra fat (Fig. 3). An increased turnover of tissue fluid and lymph *per se* did not seem to account for this effect on fat output in lymph, since, as just mentioned, larger lymph flows during intravenous infusion had little influence on the fat output. Therefore, it seemed that during increased intestinal absorption of fluid some extra fat was actually swept through the epithelial cells. This mechanical effect, if such it be, was not adequate to establish a steady state when extra fluid was given with the highest rate of fat administration, but there was a considerable and apparently sustained effect at a lower level of fat dosage.

It is tempting to speculate that there are two mechanisms for fat absorption – a metabolically active process of limited capacity, as defined by the steady state experiments, and a passive washing through of emulsified fat without metabolic intervention. From the rates observed, the former process could account for all fat absorption in rats with normal dietary and feeding habits. The latter process could explain very high fat outputs in lymph for shorter periods, after single doses of fat or at irregular intervals during rapid fat infusion. Such

speculation far outstrips the evidence available from our experiments, but has encouraged us to begin complementary experiments on absorption from the intestinal lumen.

Acknowledgments. We wish to thank Mr. N. S. Stenhouse of the Commonwealth Scientific and Industrial Research Organization for statistical analysis of the results, and Miss H. Buchan for technical assistance. We are grateful to the Medical School Research Grants Committee, University of Western Australia, for a grant towards maintenance of this research.

REFERENCES.

Bloom, B., Chaikoff, I. L., Reinhardt, W. O., Entenman, C., and Dauben, W. G. (1950). J. biol. Chem., 184, p. 1.
Bloom, B., Chaikoff, I. L., and Reinhardt, W. O. (1951). Amer. J. Physiol., 166, p. 451.
Bollman, J. L. (1948). J. lab. clin. Med., 33, p. 1348.
Bollman, J. L., Cain, J. C., and Grindlay, J. H. (1948). *Ibid.*, 33, p. 1349.
Borgström, B. (1951). Acta chem. scand., 5 (pt. 1), p. 643.
Chaikoff, I. L., Bloom, B., Stevens, B. P., Reinhardt, W. O., and Dauben, W. G. (1951). J. biol. Chem., 190, p. 431.
Deuel, H. J., Jr., Hallman, L., and Leonard, A. (1940). J. Nutrition, 20, p. 215.
Hilditch, T. P. (1947). "The Chemical Constitution of Natural Fats", 2nd ed. Wiley, New York.
Hoagland, R., and Snider, G. G. (1943a). J. Nutrition, 25, p. 295.
Hoagland, R., and Snider, G. G. (1943b). *Ibid.*, 26, p. 219.
Hughes, R. H., and Wimmer, E. J. (1935). J. biol. Chem., 108, p. 141.
Kiyasu, J. Y., Bloom, B., and Chaikoff, I. L. (1952). *Ibid.*, 199, p. 415.
Simmonds, W. J. (1954). Austral. J. exp. Biol., 32, p. 285.
Simmonds, W. J. (1955). *Ibid.*, 33, p. 305.
Stern, I., and Shapiro, B. (1953). J. clin. Path., 6, p. 158.

THE HEALING OF SURGICAL WOUNDS IN THE SKIN OF THE SHEEP

by A. K. LASCELLES AND P. MANUSU

(From the Department of Veterinary Science, University of Sydney, N.S.W.)

(*Accepted for publication 15th September, 1958.*)

SUMMARY.

The rate of healing of surgical wounds has been investigated in sheep. Sixteen incisions were placed on predetermined sites on each of three sheep. Half the wounds were closed with Michelle clips and half with transverse mattress sutures. Segments of the wounds were removed at four, six, nine and thirteen and a half days after the operation and the strength of the wounds was measured.

There was a strong linear relationship with a cubic tendency between the strength of wounds and the time after operation. Wounds closed with Michelle clips were 22 p.c. stronger than those closed with transverse mattress sutures.

INTRODUCTION.

The rate of healing of surgical wounds and the effects of various factors on this process have been studied experimentally in animals. The strength of the wound has been used as a quantitative measure of the degree of wound healing by several investigators. Harvey (1929) studied the healing of wounds in the wall of the stomach by distending the viscus with air and recording the pressure at which the wound ruptured. Howes, Sooy and Harvey (1929) measured the strength of healing wounds in the skin by means of a machine used for testing the strength of thread. Sandblom (1944) modified this machine so that more accurate measurement could be made by applying force to the wound in a chosen direction and with a relatively uniform velocity. He used this instrument to measure the breaking strength of healing wounds and studied the effect of various factors which influence this process.

The healing of surgical wounds in sheep has not been previously investigated although it is thought that this process occurs more rapidly in this animal than in others. Sheep have an extensive skin surface which is suitable for factorially designed experiments on wound healing and allows many treatment combinations to be placed on the one animal. An experiment was therefore designed to study the course of wound healing in sheep by examining the relationship between the strength of wounds and the time after operation. At

the same time, the relative efficiency of Michelle clips and transverse mattress sutures was investigated.

MATERIALS AND METHODS.

Three Merino ram lambs, which were reasonably free of body wrinkle, were closely clipped over an area which extended anteriorly to the posterior border of the scapula, posteriorly to the coxal tuber and ventrally to the costo-chondral junctions. Sixteen two-inch incision lines were marked on the skin of the clipped area with acid fuchsin. The lines ran in a dorso-ventral direction and were placed at equally spaced intervals in four rows of four (Fig. 1).

The sheep were anaesthetised with thiopentone sodium and maintained with cyclopropane in closed circuit. Each sheep was placed on the operating table so that all the incision sites were easily accessible. The experimental area was swabbed with 70 p.c. ethyl-alcohol and suitably draped. Incisions were made along the predetermined sites in random order (Fig. 1), and these passed through the full thickness of the skin and subcutaneous muscle, along their entire length. Each wound was sutured immediately following the incision.

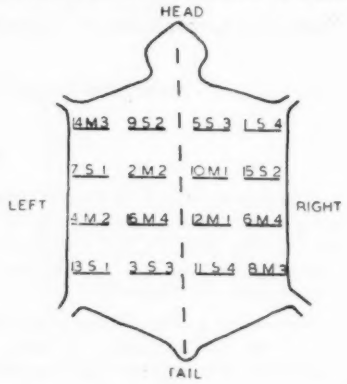


Fig. 1. Diagramatic representation of the experimental design on sheep 1. The transverse lines represent the incision sites and the number on the extreme left of each of these lines indicates the operation order. The letter M signifies that Michelle clips and the letter S that transverse mattress sutures, were used to close the respective incisions. The number (either 1, 2, 3 or 4) on the right of the incision line indicates the day after operation (either 4, 6, 9 or 13½ respectively) on which the measurements of the wound were made.

Michelle clips and transverse mattress sutures were used and these were inserted in random order (Fig. 1). The Michelle clips measured 12 mm. On the average nine clips or between six and seven mattress sutures were necessary to close each incision. A No. 8 straight, triangular pointed suture needle and No. 1 braided nylon thread were used and the thread was tied with a standard nylon knot. These sutures passed through the entire thickness of the skin approximately ⅛-in. from the edge of the wound; ⅜-in. bite was taken, and ½-in. separated each suture. After the operation each sheep was housed indoors and fed a standard stock diet.

Four wounds were removed from each sheep at four, six, nine and thirteen and a half days after the operation, these times being spaced on a logarithmic scale. Preliminary experiments had shown that skin wounds in sheep rapidly gained strength during this period. The four wounds were selected at random, restricted to the extent that each suture type was equally represented on each sheep at each time of removal (Fig. 1). The wound area was anaesthetised by subcutaneous infiltration with 2 p.c. solution of procaine (with added adrenaline). The wound, together with one half an inch margin of skin on each side, was then removed.

The wound segments were stripped completely of subcutaneous muscle immediately on removal. Three 1 cm. wide strips were cut from each wound segment and the strength of each strip was measured by an instrument which was similar in design to that used by Sandblom (1944). This breaking strength was used as the index of the extent of healing.

RESULTS.

Five of the original 144 observations were missing due to accidental tearing of part of the wound when the subcutaneous muscle was removed from the skin. For the purpose of the analysis of variance, these missing plots were completed by inserting an average of the other readings taken on that wound. The number of missing plots was subtracted from the degrees of freedom of the within-wound variance (c.f. Cochran & Cox, 1950). The analysis of variance of the experimental results is summarized in Table 1.

TABLE 1.

Summary of the analysis of variance of results of wound strength measurements in sheep.

Source of variation	D.F.	Mean square (Hectograms/cm)^2	F
Sheep	2	13·5	3·12
Times	3	122·7	28·40***
Linear	1	353·0	81·71***
Quadratic	1	1·7	0·39
Cubic	1	13·3	3·08
Suture materials	1	21·4	4·95*
First order interactions			
Sheep x time	6		
Between linear	2	1·5	0·33
Between quadratic	2	2·6	0·60
Between cubic	2	14·4	3·33*
Sheep x sutures	2	10·8	2·50
Times x sutures	3	6·7	1·55
Residual between-wound variance	30	4·32	3·29***
Measurement variance (within-wound variance)	91	1·31	

*** $P<0·001$; * $0·05>P>0·01$.

Some confusion could arise if the two sources of error were not fully appreciated. These two sources are the within-wound and between-wound variance. In calculating the variance ratio of the various sources of variation presented in Table 1, the residual between-wound variance is used as error. Within-wound variance is the term used to decide if significant correlation of results of measuring wounds exists. The F ratio is significant and the correlation between measurements on the one wound is 0·65, a satisfactorily high value (Cochran and Cox, 1950).

Fig. 2 shows the rate of wound healing during the experimental period. The analysis demonstrates a strong linear relationship between increase in wound strength and time. The between linear, sheep × times interaction is not significant, so that linear components of the regression of each individual sheep are parallel. The between cubic, sheep × times interaction is significant and has been caused by the cubic response of sheep 1 together with the flattening of the extremes found with sheep 2 and 3 (Fig. 2). Sheep 1 became listless,

slightly anorectic and febrile on the fourth day after the operation and was ill for the remainder of the experimental period.

The analysis also shows that wounds closed with Michelle clips gained strength more rapidly than wounds closed with transverse mattress sutures. The wounds closed with Michelle clips were 22 p.c. stronger and in the absence of significant interaction this finding is constant over all sheep and times of observation.

The between-wound variance was about three times greater than the within-wound variance. The between-sheep variance was approximately three times greater than the between-wound variance although this failed to reach a significant level.

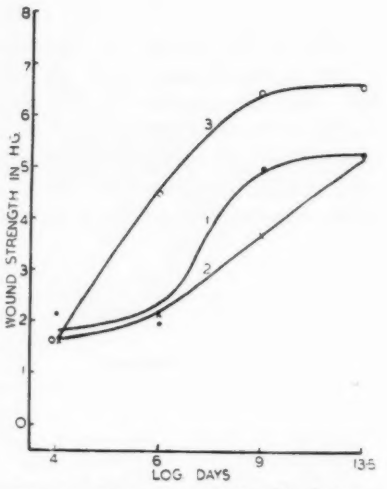


Fig. 2. Shows the relationship between the strength of the wound in hectograms (ordinate) and the time in log days (abscissa).

DISCUSSION.

Howes *et al.* (1929) using dogs and Dunphy and Udupa (1955) using rats, showed that there was an initial lag period of four to six days before wounds gained significant strength. This was followed by a phase of increasing strength with a tapering off after 10 to 14 days, the overall process following a sigmoid curve. The present investigation has indicated that the process of wound healing in sheep's skin is similar.

The more rapid healing of wounds closed with Michelle clips compared with those closed with transverse mattress sutures is probably due to differences in the amount of inflammation produced by these two methods of suturing. Michelle clips barely penetrate the skin, whereas the mattress sutures penetrate its full thickness, and any inflammatory reaction produced would delay healing (Gage and Lyons, 1949). Michelle clips have the advantage of being simple to insert but are rather too easily removed by animals. Apart from this disadvantage, it would appear that where eversion of the skin is desired, which is often the case when dealing with thin skins, Michelle clips should be chosen in preference to transverse mattress sutures.

The closeness and number of wounds may have had an effect on the overall rate of healing. Because of this possibility and to prevent any bias in the results, the randomization schedules were employed. If any alteration in the

rate of healing occurred, this would be equally shared by the other factors in the experiment.

In experiments designed to investigate the various factors which influence the rate of repair of surgical wounds, the strength of all the wounds would be best measured at the same time after the operation. The most suitable time for this measurement would be during the phase when the wounds are gaining strength most rapidly. The maximum rate of gain in strength occurred in the middle zone of the experimental period and this is the period in which treatments may be expected to show most effect. Because of the large between-animal variation it is preferable to place all treatment combinations on the one sheep and additional sheep are then used as block replicates.

Acknowledgments. We should like to thank Dr. P. J. Claringbold for his statistical advice and Dr. Bede Morris for reading the manuscript. Messrs. Hamilton and Holmes made the tensiometer.

REFERENCES.

Cochran, W. G., and Cox, G. M. (1950): Experimental Designs. Wiley: New York.
Dunphy, J. E., and Udupa, K. N. (1955): New Eng. J. Med., 253, p. 847.
Gage, I. M., and Lyons, C. (1949): Surgical Clinics of North America, 29, p. 1565.
Harvey, S. C. (1929): Arch. Surg., 18, p. 1227.
Howes, E. T., Sooy, J. W., and Harvey, S. C. (1929): J. Amer. med. Assoc., 92, p. 42.
Madsen, E. T. (1953): Surg., Gynec. Obst., 97, p. 73.
Mahoney, T. E. (1943): Amer. J. Surgery, 61, p. 414.
Sandblom, P. (1944): Acta chir. Scand., Suppl. 89.

THE SUSCEPTIBILITY OF MICE TO NORTH QUEENSLAND STRAINS OF LEPTOSPIRAE

by MARIE L. EMANUEL[1]

(From the Queensland Institute of Medical Research, Brisbane).

(*Accepted for publication 30th September, 1958.*)

SUMMARY.

Experimental infections in laboratory mice with the serotypes of leptospirae found in North Queensland could be divided into three grades: severe (two serotypes), moderate to mild (six), and inapparent (five). Survivors in the first two grades continued to excrete leptospirae in the urine probably for the rest of their lives.

Inoculation and culture of mice slightly increased the number of strains isolated from patients, as compared with culture alone, but it did not aid early diagnosis, and its use seems likely to be limited to investigations in which it is important to isolate every strain of *Leptospira* that can be obtained.

Two epidemiological implications are suggested by the work: first, that there is a pattern of relative pathogenicity of the serotypes, which may be characteristic of the species; and second, that the moderate to mild grade of infection is likely to make an animal an efficient natural reservoir of the serotypes producing it. These implications should be useful in analysing the results of field surveys.

INTRODUCTION.

Inoculation of mice with material from selected patients was a regular procedure in investigating the fevers of North Queensland at the Institute's Innisfail Field Station (17° 32′ S, 146° 02′ E). The earlier work, reported by Carley *et al.* (1955), was concerned mainly with rickettsial infections, although they recorded a few isolations of leptospirae, and noted that *L. australis A* produced much more severe infections in mice than *L. australis B*. Ross (1955) showed that blood culture was unsuccessful in about 25 p.c. of cases of leptospirosis in the area, and the present investigation was commenced to find out whether mouse inoculation would be a useful adjunct to culture in isolating leptospirae from febrile patients. It was continued in Brisbane primarily as a study of the relative pathogenicity of the different serotypes in a single animal host, in order to provide a background for the epidemiological investigations that were developing.

---

[1] Present address: Q.I.M.R. Field Station, c/o District Hospital, Innisfail, N.Q.

Materials and Methods.

Mice used. All mice used were from the Institute's stock strain, which was derived in 1949 from the Walter and Eliza Hall Institute of Medical Research, Melbourne. They are multi-coloured, relatively large, with a tendency to obesity in old age. Their weight varied considerably when they were received from the breeding room at 3 to 4 weeks of age, the mean of 500 used being 20·3 gm., with a standard deviation of 3·6 gm. There were approximately equal numbers of males and females.

It has been suggested that the use of white mice in attempts to isolate leptospirae is inadvisable, because these animals are frequently natural carriers. Wolff *et al.* (1949), for example, found that the mice from two of six breeders were infected with *L. ballum*. No leptospiral infections have ever been encountered in the Institute's stock, and all urinary and serological tests for their presence have been negative.

Inoculation from patient. Venous blood from the patient was inoculated immediately into Schüffner's or Fletcher's medium containing 20 p.c. rabbit serum, and the balance was set aside to clot. The serum was usually separated two days later, the clot then ground up with sterile alundum in physiological saline and inoculated into mice. Each mouse was given 1 ml. of emulsion intraperitoneally, and occasionally 0·03 ml. intracerebrally also.

The delay between drawing the blood and inoculating the mice could not be avoided, but it had two unfortunate consequences. The first was that the comparison was made, not between culture and mouse inoculation as methods of detecting leptospirae, but between one procedure for culture and a different, less favourable one for mouse inoculation. Secondly, it reduced the reliability of the negatives. We do not know what happens to leptospirae when lying in clot for some two days at room temperature (mean 23·6° C.). They certainly survived in many specimens, in one for five days, but we do not know whether they survived in all, nor whether their numbers may have been reduced. Three strains of *L. australis A* which failed to infect mice from the clot proved to be virulent when the culture was inoculated, suggesting that leptospirae had died during storage, and there was also an unexpected failure with a strain of *L. icterohaemorrhagiae*. A further source of error is that the importance of inapparent infections in the mice was not appreciated early in the investigation, and some may have been missed. In view of these considerations, negative results are recorded in Table 1 in brackets with a question mark.

Inoculation from patient was the procedure relied on most, because it was essential for the investigation originally planned, and because the virulence of freshly isolated pathogenic strains generally decreases quickly after repeated subculture into fluid media (van Thiel, 1948). It had the disadvantage that inoculation was blind: it was not known in advance whether viable leptospirae were in the inoculum or not, and, when they were, the infecting serotype remained unknown until cultures were identified several weeks later. It is consequently inevitable that the results obtained were unbalanced, many strains of common serotypes being investigated, and few or none of the rarer ones. There is a compensating advantage, in that ignorance of the serotype ensured a completely objective assessment of the severity of the infections it produced in mice.

Inoculation of cultures. Cultures of recently isolated strains were also inoculated into mice. The results, with serotypes for which the procedures can be compared, are shown in Table 1. There is little difference between the two sides of the table, although there may be some loss of virulence in culture by some serotypes. None in this series was in more than the second subculture, although the age from original isolation ranged from two to eight months. The dosage was 0·5 ml. intraperitoneally per mouse.

An experiment, in which 30 mice were inoculated with a first subculture of the "Robinson" serotype in order to study the course of the infection, also gave substantially similar

TABLE 1.

Pathogenicity of leptospirae to mice before and after cultivation in vitro.

Serotype	Direct from host					From recent culture				
	No. of strains	Severe	Moderate	Inapparent	Negative	No. of strains	Severe	Moderate	Inapparent	Negative
ictero-haemorrhagiae	2	—	1	—	(?1)	1	—	1	—	—
canicola	4	—	4	—	—	1	—	1	—	—
australis B	19	1	15	—	(?3)	3	1	2	—	—
"Robinson"	2	1	1	—	—	3	—	3	—	—
australis A	20	17	—	—	(?3)	5	3	2	—	—
grippotyphosa	1	1	—	—	—	1	1	—	—	—
kremastos	5	—	—	5	—	4	—	—	—	4

results to those following direct inoculation. On the other hand, the survival rate in *L. australis A* infections was much higher in mice inoculated with cultures (48/60) than in those that had been infected from clot (1/18), although the general course of infection and lesions observed were similar in the two series.

Another experiment was therefore set up to test the virulence of *L. australis A* during cultivation. Two strains, derived from human cases, were grown on both Schüffner's and Fletcher's media, and the pathogenicity of each series was tested at regular intervals by inoculating six mice with the standard dose of a six to seven-day-old subculture. The results (Table 2) indicated that there was little loss of virulence by these strains, even after nine to 12 months cultivation, for each series except the first still produced some mortality in the mice.

TABLE 2.

Virulence of L. australis A *for mice during cultivation* in vitro.

Strain and original medium	Initial infections in mice	Subcultured in	Final age since isolation	Number of subcultures	Infections at end of experiment
McL. Fletcher	Severe	Schüffner	370 days	18	moderate
		Fletcher	356 days	19	severe
W. Schüffner	Severe	Schüffner	265 days	19	severe
		Fletcher	265 days	21	severe

In view of all these findings, the pathogenicity tests with recent cultures have been incorporated in the results reported below, but they have been used with caution, and with the particular reservation that they are probably not reliable with strains of inherently low pathogenicity.

The foregoing does not apply to old stock strains, maintained by weekly subculture in Schüffner's medium. Batches of from ten to 40 mice were inoculated with young subcultures from the stocks of each of 13 serotypes known from North Queensland, the periods from original isolation varying from eight months to 27 years. Leptospirae could be demon-

strated in the mice, by culture or dark-ground examination of peritoneal fluid or blood, for from one to five days, but no macroscopic lesions or other evidence of infection could be detected at any stage.

Examination of the mice. Inoculated mice were examined daily for signs of illness and some of the series were weighed regularly. Occasionally, peritoneal puncture was performed in the early stages, and the fluid searched for leptospirae under dark-ground illumination.

Mice that were sacrificed were examined by a regular procedure. Fresh sterile instruments were used for each stage of the dissection, blood for culture was drawn by heart puncture as soon as the skin had been reflected from the chest, and tissues for culture were obtained immediately the body-cavity was opened, by snipping off small pieces with fresh sterile scissors and dropping them into the medium. The organs were then examined for macroscopic lesions, particular attention being paid to haemorrhages in the lungs or connective tissue, jaundice, enlargement of lymph glands, and the appearance of the liver and kidneys. Material for dark-ground examination was obtained from liver and kidney by scraping the cut surface with a sterile platinum loop, and rubbing up the material obtained in one or two drops of saline on a glass slide. A 22 mm. square cover-slip was applied, and the whole area searched for leptospirae. An 8 mm. objective was found useful for this work.

Mice that had died were treated similarly, if their condition permitted, but the examination was usually limited to a record of the macroscopic lesions present.

RESULTS.

It became apparent, as the work progressed, that infections in the mice could be divided into three fairly distinct grades:

Severe, with obvious illness, obvious macroscopic lesions in internal organs, high mortality, and survivors becoming chronic urinary excretors of leptospirae.

Moderate to mild, with few or no clinical signs, some evident macroscopic lesions, and usually ending in recovery and continued excretion of leptospirae in the urine.

Inapparent, with no clinical signs and usually no detectable lesions, but usually ending in at least some of the mice becoming urinary carriers.

It will be convenient, for clarity of presentation, to arrange the serotypes in these grades, rather than according to their serological classification.

Severe infections.

L. australis A. Clots from 20 patients were inoculated into mice. Three failed to infect. All the 47 mice inoculated with the remaining 17 suffered illness: 29 were killed for examination between the 6th and 12th days; 17 died between the 8th and 18th days; one survived, and was still excreting leptospirae when killed on the 489th day after inoculation.

Infected mice appeared ruffled and hunched about five days after inoculation, and became progressively more distressed until death, which occurred most often on the 9th day (two on 8th, six on 9th, three on 10th, three on 11th, two on 12th, one on 18th). There was definite loss of weight in all series that were weighed, as illustrated by the charts in Fig. 1. Mice were generally very

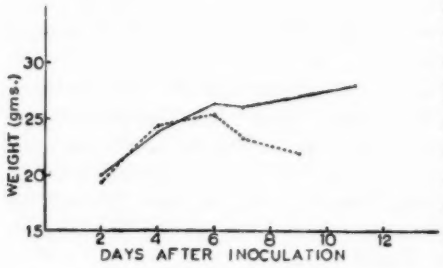


Fig. 1. *Weight charts of mice inoculated on the same day with blood clot from two patients.* Each line the mean of three mice.
. ——. Received uninfected blood.
. — —. Received blood infected with *L. australis A.*

feeble and tremulous for one or two days before death, and occasionally were partly paralysed in the hind legs. Jaundice was noted often in the ears, footpads and tail. Epistaxis was a frequent sign in animals which succumbed to the disease, and also in moribund animals when administered anaesthetic.

The following lesions were noted on post-mortem examination of sick or dead mice: congestion of the subcutaneous tissue, generally with multiple small haemorrhages; haemorrhagic areas in the lungs, in the inguinal and axillary regions, and occasionally in limb muscles, chest wall and peritoneum; glandular enlargement, most noticeable in the inguinal and axillary glands; generalised jaundice in some mice; pale liver; and extremely pale, often yellowish kidneys. Leptospirae were demonstrated, by culture or dark-ground examination, from the 6th to the 10th day in blood (in 12 of 13 mice examined), liver (4/18), kidney (21/27), and were grown from the kidney of the survivor killed on the 489th day; they were also seen on the 6th and 7th days in peritoneal fluid (5/5).

More detailed information about the course of infection was obtained from another experiment, in which 20 mice were inoculated with a young subculture of one of the virulent strains referred to above, and one or more killed each day for the first six days, and thereafter at 8, 10, 14, 20, 28 and 37 days after inoculation.

The first macroscopic signs of infection noted were haemorrhages in the lungs of two mice autopsied at two days. At three days, the animal sacrificed presented, in addition to the lung haemorrhages, small subcutaneous and peritoneal haemorrhagic lesions and some glandular enlargement. One animal killed at ten days showed some evidence of regressing haemorrhagic areas in the axillary and inguinal subcutaneous tissue, but no lung haemorrhages. Little glandular enlargement or other abnormality could be detected in mice sacrificed after 14 days, except for the kidneys, which were paler than normal.

Some bias was introduced in this experiment during the four to ten-day period, in that the animals that appeared distressed were sacrificed for examination, leaving those which did not readily succumb for later examination. Three animals died, one at four and two at six days. When examined for the first time at 13 days, urines of three animals showed numerous leptospirae.

The lesions in these and other mice inoculated with cultures were exactly

similar to those in mice inoculated with clot, the only difference being that they ceased to progress during the second week, and most of the animals recovered. All the mice examined (31) had become chronic urinary carriers.

L. grippotyphosa. Only one human infection with this serotype has been recorded in Australia (Smith and Brown, 1956). Clot from the patient produced severe reactions in two mice, one of which was killed on the 8th day, and the other died on the 10th. The lesions were similar to those produced by *L. australis A*, and leptospirae were seen in the kidney on the 8th day. The culture was also virulent. Of ten mice inoculated, five died, four were killed, and the one survivor became a chronic urinary carrier.

These results seem to be rather exceptional. Van Thiel (1948), in reviewing the literature, stated that guinea-pigs were more susceptible to mud fever than laboratory mice. Other strains of *L. grippotyphosa* have been reported as non-pathogenic (Bernkopf *et al.*, 1948) or mildly pathogenic (Borg-Petersen, 1950) to mice.

Moderate to mild infections.

L. icterohaemorrhagiae. This is an uncommon serotype in North Queensland, Derrick (1957) recording only ten infections due to it among 404 cases of leptospirosis he reviewed. Clots from two patients were injected into mice. One failed to infect. The other produced moderate infections in two mice. When killed on the 11th day, they showed haemorrhages in the lungs, swollen lymph glands, and pale kidneys containing live leptospirae. A culture of this strain also produced moderate infections in ten mice, with no deaths, three out of the four not killed for examination becoming urinary carriers.

The mouse is the experimental animal which has been used, along with the guinea-pig, for the demonstration of virulent or moderately virulent *L. icterohaemorrhagiae*. The literature has been reviewed by Walch-Sorgdrager (1939) and van Thiel (1948).

L. canicola. Four strains from clots and one from culture were inoculated into 20 mice. They produced infections which were indistinguishable, in course and signs at autopsy, from those described below for *L. australis B*; surviving animals became urinary carriers.

Walch-Sorgdrager and Meyer *et al.* (1939) recorded inapparent disease, followed by urinary infection in mice. In North Queensland, this serotype seems to be associated with wild animals rather than dogs, in which serological evidence of infection was obtained in only one of 33 examined (unpublished data). The serological relationships of all human strains inoculated into mice and two strains isolated from marsupial bandicoots (*Thylacis* sp.) characterise them as the "complete biotype" of *L. canicola* (Smith, personal communication).

L. australis B. Clots from 19 patients and cultures from three (including two also examined by clot) were inoculated into mice. Three clots apparently

failed to infect (the mice were not examined in sufficient detail to exclude mild or inapparent infections). One strain produced severe infections, both from clot and culture, and the other 16 produced moderate to mild infections.

Of four mice inoculated with clot containing the strain which produced severe infections, one was killed on the 6th day, one on the 9th, one died on the 11th, and the fourth was killed when moribund on the 12th. Clinical signs, macroscopic lesions, and recovery of leptospirae from blood, liver and kidneys were similar to those observed in *L. australis A* infections. Of 12 mice inoculated with cultures of this strain, three died, and nine became chronic urinary carriers.

There were no deaths attributable to leptospirosis in the 40 mice inoculated with clots and four with cultures of the other 16 strains. Clinically, they showed ruffled coats and appeared mildly distressed within a few days. These signs of infection could be noted only after keen daily observation, and were not as severe as those shown by mice infected with the virulent strains discussed earlier. Mice not sacrificed for investigation or passage made a complete recovery within about ten days, and became healthy carriers. Generally no loss of weight was demonstrated in experiments in which daily weights of animals were recorded.

Signs of leptospiral infection were detected readily in mice sacrificed about six days after inoculation, but few lesions were seen when animals were examined at ten to twelve days. Jaundice has never been noted in mice infected with *L. australis B*. Haemorrhages in the lungs and subcutaneous tissue, present in animals examined in the early stages of infection, were generally not severe, and (except in the severe case) the very pale, haemorrhagic kidneys characteristic of virulent *australis A* infections were not observed.

Splenomegaly was occasionally present in mice infected with *L. australis B*. The spleen is considered to be enlarged, when the product of the length and breadth in millimetres is greater than 100 (Derrick *et al.*, 1954). In this series, six out of 35 mice necropsied between 6 and 12 days after inoculation had splenic indices between 102 and 126. Splenomegaly has not been seen in mice sacrificed several weeks or months after they had become urinary carriers.

Leptospirae were demonstrated, by culture or dark-ground examination, in the blood on the 6th to 7th days (in 5 out of 5 mice examined), peritoneal fluid on the 6th to 7th days (3/3), liver on the 6th to 9th days (3/7), and kidney (16/18) on the 6th to 9th, 43rd, 52nd, 70th, 86th and 383rd days after inoculation. All 14 recovered mice, whose urine was examined, had become chronic carriers.

"Robinson" serotype. This serotype is closely related antigenically to *L. australis B*. Three strains have been investigated, two from both clot and culture, and the third from culture only. One strain from clot produced severe infections in four mice, of which two were killed at 7 and 9 days, and the other

two died on the 11th day. Signs were like those of an *L. australis A* infection, and leptospirae were recovered from the blood, liver and kidney on the 7th day. The other strain from clot produced moderate infections in two mice. One showed splenomegaly, swollen glands, and pale kidneys containing leptospirae, when killed on the 15th day; the other was still excreting leptospirae, when it died of a lung infection 168 days after inoculation.

Cultures of all three strains (including the more virulent one) produced moderate infections, with no deaths attributable to leptospirosis, in the 64 mice into which they were inoculated. The animals were not severely distressed, and showed no jaundice, but lesions suggestive of leptospirosis were present in those autopsied in the two to seven day period; at least 29 of the 33 survivors became urinary carriers.

"Esposito" serotype. This serotype is closely related antigenically to *L. australis A.* A strain was recovered in guinea-pigs from "trash" gathered in the canefield where the original patient had been working (Doherty, Emanuel and Moore, 1956). Four mice, inoculated with livers of first and second passage guinea-pigs, developed moderate infections, with evident lesions in two of the three killed for examination, and the fourth surviving as a chronic urinary carrier. A ten-month-old culture from the patient, which had been passaged three times through culture media, was non-pathogenic to the six mice into which it was injected.

L. hyos. Clots from two patients produced mild infections in six mice. There were no clinical signs, but two mice killed on the 7th and 10th days showed some glandular enlargement, subcutaneous engorgement, "butterfly" lungs, and leptospirae in their kidneys. The four survivors became urinary carriers, still having leptospirae in their kidneys when killed 102 days, 19 months, and 2 years (two animals) after inoculation.

Inapparent infections.

L. kremastos. This member of the *hebdomadis* group was named by Wolff and Bohlander (1958). Clots from five patients were tested in 17 mice. None developed any clinical indication of infection, but there was slight glandular enlargement in four of seven killed between the 5th and 10th days, and some subcutaneous congestion in one. Leptospirae did not grow in any of the six cultures of heart blood made between the 5th and 10th days, but they were isolated from one (on the 5th day) of the five livers. They were also demonstrated in the kidneys of seven mice killed on the 5th, 6th, 8th, 28th, 66th, 86th and 163rd days after inoculation, and intermittently for four to six months in the urine of four others. Thus, all the strains were shown to infect some of the mice into which they were inoculated, but proof of infection depended almost entirely on finding leptospirae in their urine or kidneys.

It was difficult to demonstrate the carrier state in mice infected with this serotype. Excretion of leptospirae seemed to be irregular, the routine method

of examining the urine often gave negative results, and, when the organisms were found, their numbers were fewer than were usually seen in infections with the more virulent serotypes.

Cultures of four strains, including three discussed above, were inoculated into 96 mice. They produced a transitory leptospiraemia, of three to four days duration, with organisms also in the peritoneal fluid, but no other evidence of infection, and (except for a doubtful record in one mouse) no leptospirae were found at any time in the kidneys or urine. It would seem that the ability of *L. kremastos* to parasitise mice is easily lost.

L. celledoni. This serotype was described by Broom and Smith (1956). Clots from four patients were inoculated into ten mice. One mouse was lost; five, killed between the 4th and 10th days, were recorded as negative, but were not fully investigated for inapparent infections; one, killed on the 6th day, showed slight haemorrhages in the lungs and doubtful subcutaneous congestion; leptospirae were recovered from a second passage mouse inoculated from one killed on the 7th day, and were seen on the 13th and 43rd days in the kidneys of two others. In sum, two of the strains produced inapparent infections, and two may have failed to infect.

Unallotted

L. pomona. A culture of a bovine strain, which had been through seven guinea-pig and two culture passages, was inoculated into ten mice. They showed no signs of infection, other than slight glandular enlargement in four of five killed in the first 12 days, but four of five survivors became urinary carriers. A three months old primary culture from a patient produced no infections in 20 mice, other than transitory leptospiraemia, like that produced by stock cultures.

Clayton, Derrick and Cilento (1937) found no sign of infection in four white mice inoculated with a culture of *L. pomona,* and Mochtar (1940) found that none of six strains isolated from pigs in Batavia was highly virulent for white mice. Thus, the observations reported here may be normal, but a study of strains which have not been through culture media or laboratory animals would be desirable, before placing this serotype in the sequence of pathogenicity.

"Szwajizak" strain. This strain was confirmed by Wolff and Bohlander as an incomplete biotype of *L. mini* Babudieri. Clot from one patient was inoculated into two mice, which developed no evidence of infection (other possibly than a splenic index of 120 in one), but they were not fully examined for inapparent infections. Cultures from another two patients produced nothing beyond a transitory leptospiraemia in 40 mice, in which late renal infections were adequately searched for.

L. medanensis. Clot from one patient was injected into two mice early in the investigation. They were recorded as negative, but had not been fully examined for inapparent infections.

DISCUSSION.

The use of mouse inoculation in diagnosis.

The overall results (Table 3) show that mouse inoculation failed in 11 cases in which culture was successful, and succeeded in six in which culture failed. The failures were: *australis A* 3, *icterohaemorrhagiae* 1, *australis B* 3, *celledoni* 2, *mini* 1, and *medanensis* 1. The first seven were probably attributable to the delay in inoculating the mice, and the last four to weak pathogenicity and incomplete search for inapparent infections. The successes were: one each of *australis A*, *icterohaemorrhagiae*, *australis B*, *hyos*, *kremastos* and *celledoni*. Failure of the cultures seems most often to have been due to contamination.

TABLE 3.

Comparison of mouse inoculation and cultures from 86 patients with leptospirosis.

Infections in mice	Leptospirae cultivated from blood	Leptospirae not cultivated from blood	Total
Positive	45	6	51
Not proved, or negative	11	24*	35
Total	56	30	86

* Diagnosed by rising antibody titres in sera.

Considered simply as an adjunct to culture, inoculation of the mice increased the isolations from 56 (65 p.c.) to 62 (72 p.c.) of the 86 cases studied, but it uncovered no serotypes that culture failed to detect. Using suckling mice, McCrumb *et al.* (1957) concluded that Fletcher's medium was twice as effective for isolation of leptospirae as their method of isolation from the brains of the animals. Our results might have been improved by inoculating the mice at the bedside at the same time as the media, but that cannot often be done in practice, and it was not attempted here. Even as it stands, the method would be useful in any investigation in which it is important to isolate every strain of *Leptospira* that can be obtained, but it would be necessary to ensure that the mice used were free from natural infections.

The position is different from the point of view of clinical diagnosis. In common with other laboratory animals which have been utilised by various workers, it was found, with our mouse strain, that the value of mouse inoculation depended largely on the serotype involved, and that a diagnosis in time to influence treatment would generally not be possible. The lesions seen in weaned mice killed during the acute stage of severe or moderate infections are distinctive, but they are not distinctive in many mild or inapparent infections. It is essential to demonstrate the presence of leptospirae, and it is worth emphasising in this connexion that there are two stages in an infection at which they can be

found most readily—the blood and peritoneal fluid from the fourth to seventh days, and the urine and kidneys from the latter part of the second week onwards. The latter is particularly important in inapparent infections. In practice, a retrospective diagnosis can usually be made more simply by serological methods when culture fails.

It would help the clinician materially, if leptospirae could be found more quickly in inoculated mice than in culture. Popp (1950) stated that they could be seen in peritoneal fluid of mice as early as two days after inoculation of citrated blood. Only a few observations were made on the mice inoculated with clot. No leptospirae were found on the second day in the peritoneal fluid of three mice infected with *L. australis B* and one each with *L. australis A, L. kremastos* and the "Robinson" serotype, but they were seen on the fourth day in two of three mice with *L. australis B* and one with *L. australis A*. Leptospirae can be found regularly in the peritoneal fluid 24 or 48 hours after inoculation of large numbers in cultures, so differences in dosage by the two methods may explain the discrepancies in results.

An interesting sidelight on the use of mice was the aid they gave in confirming the double infection with *L. hyos* and *L. mini* reported by Smith and Doherty (1956). Leptospirae were not isolated from the two mice inoculated with the patient's blood, although they were seen in the kidney of one, but antibodies to both serotypes were found in the sera of second passage mice.

Epidemiological implications.

Two inferences of basic significance in epidemiology are suggested by this work. One is, that there is a pattern of pathogenicity of the different serotypes that may be characteristic of the host species, and the other, that there is a particular level of pathogenicity that is most likely to make an animal a good reservoir of leptospirae in nature.

The general experience that serotypes vary greatly in pathogenicity is well expressed in the statement by Wolff (1954) that no laboratory animal which is universally susceptible for all known strains of pathogenic leptospirae has yet been found. If we include the unallotted types with the inapparent infections, where they seem most likely to belong, the pattern of pathogenicity of the Australian serotypes in our strain of laboratory mice can be epitomised in the following statement:

Severe	Moderate to Mild	Inapparent
australis A	*icterohaemorrhagiae*	*pomona*
grippotyphosa	*canicola*	*kremastos*
	australis B	*mini*
	"Robinson"	*medanensis*
	"Esposito"	*celledoni*
	hyos	

It is not suggested that this grading is immutable. Some of the serotypes may not be correctly placed – for example, *L. icterohaemorrhagiae* may really belong in the severe group, and *L. hyos* with the inapparent infections. Moreover, different strains of a single serotype may vary in pathogenicity, as was seen in the *australis B* and "Robinson" serotypes. Also, pathogenicity of single strains may alter, as occurs in culture, and we do not know enough about the qualities which make leptospirae virulent, or the circumstances which induce changes in them, to prophesy what may happen in nature.

Packchanian (1940) showed that there are differences of varying degree in the susceptibility of different species of mice and rats to leptospirosis. Stavitsky and Green (1945) suggested that "in addition to differences between species, there are differences within species". Neghme *et al* (1951) concluded that the genetic constitution of mice is an important factor in their susceptibility to experimental leptospirosis. In spite of these reservations, the general uniformity of the results obtained with the individual serotypes induces quite a strong feeling that the above list does give a broadly accurate picture of a broadly stable situation.

Evidence from field investigations, and from published work on other laboratory animals and man, supports the hypothesis that there is a characteristic pattern of pathogenicity in other host species, and that this differs from species to species. It would be highly desirable to test the hypothesis further by repeating the present work with at least one species of rat, and, if possible, with a marsupial.

The second inference is evident from the facts set out in the body of the paper. Animals are not likely to be good reservoirs of organisms that kill them, and it may be doubted, too, whether they would be good reservoirs in nature for organisms which produce such tenuous, even if sometimes persistent, infections as are recorded in the inapparent group. On the other hand, the moderate and mild infections do not often make the animal so ill that its chances of surviving in nature seem likely to be seriously reduced, and the constancy and persistence of the resultant urinary carrier state is a most remarkable feature. Indeed, it seems likely that every mouse surviving an infection of this grade continues to excrete leptospirae in large numbers for the rest of its life. This is an ideal host relationship for an organism which is spread through soil contamination.

Acknowledgments. The identification of leptospiral strains was carried out by the Laboratory of Micro-Biology and Pathology, Department of Health and Home Affairs, Brisbane. The writer is indebted to Dr. I. M. Mackerras and Dr. E. H. Derrick for their generous help with all aspects of this work, and with the preparation of this paper. She is also indebted to Dr. R. L. Doherty and Mrs. D. G. Delamoir for their assistance with animal inoculation; and to Mrs. M. Macgregor, librarian of the Queensland Institute of Medical Research, for help with references.

REFERENCES.

Bernkopf, H., Olitzki, L., Stuczynski, L. A., Gotlieb, T., and Halevi, C. (1948): J. infect. Dis., 83, p. 232.
Borg-Petersen, C. (1950): Acta path. microbiol. Scand., 27, p. 726.
Broom, J. C., and Smith, D. J. W. (1956): Lancet, 2, p. 866.
Carley, J. G., Doherty, R. L., Derrick, E. H., Pope, J. H., Emanuel, M. L., and Ross, C. J. (1955): Austral. Ann. Med., 4, p. 91.
Clayton, G. E. B., Derrick, E. H., and Cilento, R. (1937): Med. J. Austral., 1, p. 647.
Derrick, E. H. (1957): Papua and New Guinea med. J., 2, p. 23.
Derrick, E. H., Pope, J. H., Chong, S. K., Carley, J. G., and Lee, P. E. (1954): Austral. J. exp. Biol., 32, p. 577.
Doherty, R. L., Emanuel, M. L., and Moore, N. (1956): Med. J. Austral., 1, p. 276.
McCrumb, F. R., Jr., Stockard, J. L., Robinson, C. R., Turner, L. H., Levis, D. G., Maisey, C. W., Kelleher, M. F., Gleiser, C. A., and Smadel, J. E. (1957): Amer. J. trop. Med. Hyg., 6, p. 238.
Meyer, K. F., Stewart-Anderson, B., and Eddie, B. (1939): J. Amer. vet. med. Assoc., 95, p. 710.
Mochtar, A. (1940): Geneesk. Tijdschr. Ned.-Ind., 80, p. 2334. Bull. Hyg., Lond., 16, p. 175 (1941).
Neghme, A., Christen, R., Jarpa, A., and Agosin, M. (1951): Bol. parasit. chil., 6, p. 4.
Packchanian, A. (1940): Pub. Hlth. Rep., Wash., 55, p. 1389.
Popp, L. (1950): Zentralbl. Bakt., 1 Abt. Orig., 155, p. 221.
Ross, C. J. (1955): Med. J. Austral., 2, p. 319.
Smith, D. J. W. (1958): Personal communication.
Smith, D. J. W., and Doherty, R. L. (1956): Med. J. Austral., 2, p. 643.
Stavitsky, A. B., and Green, R. G. (1945): Science, 102, p. 352.
van Thiel, P. H. (1948): "The Leptospiroses", Leiden, University Press.
Walch-Sorgdrager, B. (1939): Bull. Hlth. Org. L.o.N., 8, p. 143.
Wolff, J. W. (1954): "The Laboratory Diagnosis of Leptospirosis", Thomas, Springfield, Ill.
Wolff, J. W., and Bohlander, H. J. (1958): Trop. geogr. Med., 10, p. 37.
Wolff, J. W., Bohlander, H., and Ruys, A. C. (1949): Leeuwenhoek ned. Tijdschr., 15, p. 1. Bull. Hyg., Lond., 24, p. 963 (1949).

REMOVAL OF HAEMOGLOBIN FROM THE BLOOD OF NORMAL AND NEPHRECTOMISED RATS

by ILSE BRADING, SHIRLEY A. CANTRILL AND R. J. WALSH

(From the New South Wales Red Cross Blood Transfusion Service, Sydney).

(*Accepted for publication 10th September, 1958.*)

SUMMARY.

The removal of Fe^{59}-labelled haemoglobin from the blood stream has been investigated in normal and nephrectomised rats. The kidney takes up a large part of the haemoglobin in intact animals, but small amounts are also removed by the liver and spleen. In the absence of the kidney, the liver, spleen and bone marrow remove proportionately greater amounts of haemoglobin so that eight hours after injection the concentration in the blood is the same as that in the intact animals.

INTRODUCTION.

The kidney appears to be the organ normally concerned with the clearance of extra corpuscular haemoglobin from the blood (Yuile, 1942; Finch, Thomas, Walsh and Fluharty, 1948). When the level of haemoglobin in the blood exceeds a low threshold, it passes through the glomeruli into the renal tubules. The haemoglobin is absorbed by the cells of the proximal convoluted tubules (Rather, 1948; Black, 1948) where it is slowly degraded to produce ferritin and stainable haemosiderin. The appearance of haemoglobin in the urine depends on the rate of glomerular filtration and on the absorptive capacity of the tubules.

In man, intravascular haemolysis sometimes occurs before and during acute renal failure. When the kidneys cease to function haemoglobin cannot be removed from the blood by the kidneys, and must be eliminated in some alternative manner, the mechanism of which is unknown. It was thought that this pathway should be investigated, especially as its elucidation might assist in the management of patients with intravascular haemolysis and acute renal failure, such as occurs in some incompatible blood transfusions, in infections with *Clostridium welchii* and occasionally with myohaemoglobinuria following crush injuries.

A solution of Fe^{59}-tagged haemoglobin was injected intravenously into two groups of rats. The method of preparation and injection was similar to that of Finch *et al.* (1948). The first group consisted of normal animals and the second of animals from which both kidneys had been removed 24 hours previously. Representative animals from each group were killed at various intervals after

injection of the solution and the Fe^{59} in the blood and various organs was measured. The results show that in the absence of functioning kidneys, the rat has an efficient alternative method of removing haemoglobin liberated into the blood stream.

MATERIALS AND METHODS.

Experimental animals.

Male albino rats weighing between 190 and 430 gm. were divided into two series each of 28 rats. The first series with a mean weight of 317 gm. was used as control and all in the second series with a mean weight of 311 gm. were nephrectomised. Five large rats weighing approximately 400 gm. were used as donor rats.

Preparation of radioactive haemoglobin.

A solution of $Fe^{59}Cl_3$ was obtained from A. E. R. E. Harwell, through the agency of the Commonwealth X-Ray and Radium Laboratory, Melbourne. Each of the five donor rats was injected intravenously with 20 μ.c. of the radioactive iron solution in 0·3 ml. Three weeks later blood was obtained from each animal. Under ether anaesthesia the chest was opened and blood taken from the heart into a heparinised syringe.

The red cells were separated by centrifugation and haemolysed by freezing after the addition of an equal volume of distilled water. The solution was thawed, and sufficient 17 p.c. saline solution added to bring the salt concentration to 0·85 p.c. The stroma was separated by centrifugation and the supernatant solution used for injection.

Nephrectomy.

The animals were anaesthetised with ether and the kidneys exposed through incisions parallel to the costal margins in the region of the costo-vertebral angles. The renal vessels and the ureter on each side were clamped with curved mosquito artery forceps and ligated with cotton thread passed around the kidney and forceps. The vessels were divided close to the kidney which was then easily removed. The muscle layer and the skin were separately sutured with cotton.

Administration of haemoglobin solution.

On the day after nephrectomy, one millilitre of the haemoglobin solution was injected through a fine needle into the heart of each rat of both series.

Collection and assay of specimens.

The two series were divided into four groups each of 7 rats, and a group from each series was killed at time intervals of 2 hours, 4 hours, 8 hours and 24 hours after injection of the haemoglobin. Under ether anaesthesia blood was collected from the heart, and, before removal, the liver and spleen were perfused with physiological saline as previously described (Brading, George and Walsh, 1956). Both femurs of each animal were removed in addition to the livers and spleens of all animals, and the kidneys in the control series. The haemoglobin values of the blood samples were measured by the method of Walsh *et al.* (1953). After the volume was measured the blood was transferred to a specimen container for radio-assay. Distilled water was added to give a total volume of 20 ml. and a small amount of saponin added to ensure complete haemolysis of the red cells.

The livers, spleens and kidneys were homogenised in a cylinder and rotating piston type of homogeniser. The homogenate was transferred to a specimen container and the volume made up to 20 ml. with physiological saline. In previous work, animal tissues had been digested with sulphuric and perchloric acids prior to radio-assay. This was found to be unnecessary because the tissue homogenates gave similar results. The femurs were cleaned of tissue and placed on the bottom of specimen containers.

The samples were assayed using an "EKCO" scintillation counter and scaler. The specimen containers were placed on one face of a thallium-activated sodium iodide crystal, 1¼ inches in diameter and one inch long, and the impulses were registered on the scaler in the conventional manner. The results were converted to counts per minute for each of the tissues assayed. The blood volume was calculated on the basis of 6·7 ml. per 100 gm. body weight and from this value and the volume of blood assayed, the activity present in the blood was obtained.

RESULTS.

The results are shown in Table 1 and illustrated graphically in Figs. 1 and 2. In intact animals a large portion of the haemoglobin is removed from the blood within two hours of injection. The rate of removal is still rapid during

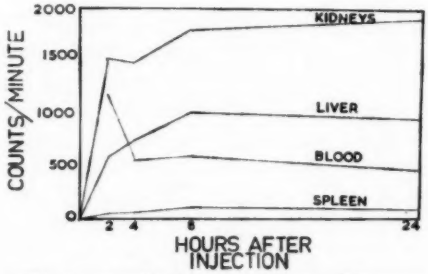


Fig. 1. Localisation of Fe^{59}-tagged haemoglobin in animals with intact kidneys, expressed as total counts per minute in whole organ.

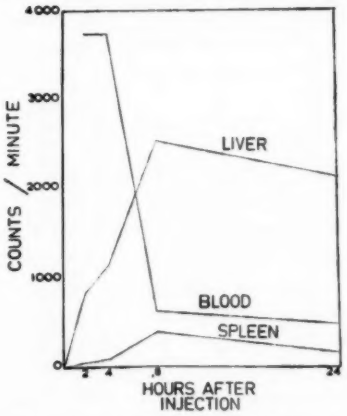


Fig. 2. Localisation of Fe^{59}-tagged haemoglobin in animals with both kidneys removed, expressed as total counts per minute in whole organ.

the next two hours, but thereafter becomes slower. Significant amounts remain in the blood at 24 hours. The predominant rôle of the kidney is obvious and the uptake by the organ is greatest in the first two hours. At 24 hours the kidneys contain only 33 p.c. more than they did at two hours. On the other hand, the maximum concentration in the liver and spleen is not reached for 8 hours, and even at 24 hours the total amount of Fe^{59} in these tissues is not as great as in the kidneys.

In the nephrectomised animals the rate of clearance of haemoglobin from the blood is slower but at 8 hours the level in the blood is approximately the same as in the intact animals at this time. Corresponding to this clearance rate the uptake by the liver and spleen increases to a greater extent in the two to eight hours period than in the intact animals, and is maximal at 8 hours. The amount of Fe^{59} in the liver and spleen at 8 hours is about 3·5 times that present

at 2 hours. Nevertheless, the liver and spleen contain approximately the same amount at 8 hours as was found at this time in the liver, spleen and kidneys combined in the intact animals. In the nephrectomised animals the liver takes up a greater total amount of haemoglobin than the spleen, but both organs are involved in the removal.

TABLE 1.

Fe^{59} in tissues following injection of labelled haemoglobin solution into (A) control intact animals and into (B) animals with both kidneys removed.

Experiment	Time after injection (hours)	Fe^{59} as mean counts per minute (c.p.m.)							
		Blood		Liver		Spleen		Kidneys	
		Total	per ml	Total	per gm.	Total	per gm.	Total	per gm.
A	2	1203[1] ±423	50	589 ± 75	46	51 ± 4	66	1504 ±347	537
	4	528 ±158	29	718 ± 47	72	60 ± 12	75	1494 ±136	709
	8	558 ±167	28	969 ±120	88	110 ± 19	129	1766 ±752	728
	24	487 ± 96	22	904 ± 32	74	103 ± 9	117	1959 ±825	686
B	2	3775 ±245	198	705 ± 48	80	61 ± 4	86	—	—
	4	3755 ±633	172	1005 ±140	92	106 ± 15	133	—	—
	8	624 ±142	27	2492 ±1047	235	319 ± 55	447	—	—
	24	506 ± 40	26	2385 ±116	235	243 ± 29	382	—	—

[1] Standard error of the mean is obtained from $\frac{\delta}{\sqrt{n-1}}$ where n is the number of observations.

Table 1 also shows the concentration of Fe^{59} in counts per minute per gram of tissue and these values are presented graphically in Fig. 3. In the intact animals the liver and spleen have approximately equal values, but when the kidneys are removed the concentration of Fe^{59} in the spleen increases to a greater extent than in the liver.

The amount of Fe^{59} in the femurs, as shown in Table 2, is surprisingly large. At 2 and 4 hours it is approximately the same

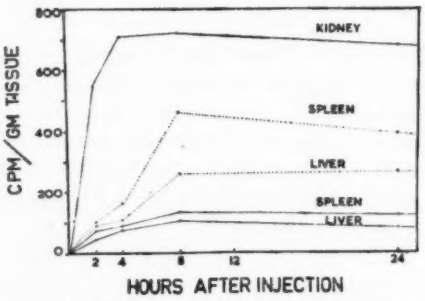


Fig. 3. Localisation of Fe^{59} in organs, expressed as counts per minute per gram of tissue. Continuous lines show results obtained on animals with kidneys intact; broken lines show results obtained on animals after removal of kidneys.

TABLE 2.

Mean radioactivity (c.p.m.) of both femurs.

Hours	Animals	c.p.m.	Animals	c.p.m.
2	Controls	93	Nephrectomised	100
4	Controls	79	Nephrectomised	99
8	Controls	137	Nephrectomised	195
24	Controls	122	Nephrectomised	195

in both series, but at the later periods is greater in the nephrectomised group. Since the femurs contain only a small proportion of total bone marrow in the body, it is apparent that bone marrow tissue takes up a significant amount of haemoglobin from the blood. However, the femurs were radio-assayed in the intact state with contained blood, and therefore the results are not strictly comparable with those in Table 1.

DISCUSSION.

It appears that haemoglobin is removed from the blood in two ways. The first is by the kidneys, both by absorption and by excretion. The second is by the liver, spleen and bone marrow, and occurs in the intact animal but exclusively in the nephrectomised animal. The second pathway responds to increased demand after an initial lag of some hours, but the physical mechanism of haemoglobin uptake is not known. The findings suggest that even with intact animals there is active removal of haemoglobin by the liver and spleen and not merely a transfer of Fe^{59} from the haemoglobin which has been broken down in other organs. Jandl, Richardson Jones and Castle (1957) noted that the liver and spleen removed labelled haemoglobin from the blood of normal subjects.

Both mechanisms probably operate in human patients with intravascular haemolysis and superimposed acute renal failure. In the initial stages, haemoglobinuria occurs and renal tubular absorption removes some haemoglobin. Later when the kidneys cease to function the haemoglobin is eventually removed by other organs. A delay of a few hours results in the formation of methaemalbumin in the blood (Fairley, 1941). The practical point arising is whether persistent intravascular haemoglobin or methaemalbumin aggravates the renal damage and whether active measures should be instituted to remove the material from the blood. Some workers doubt whether haemoglobinaemia *per se* is either directly or indirectly involved in the pathogenesis of renal failure, but this point has not been investigated in the present experiments. The second method of haemoglobin removal shown by the current experiments is, however, efficient and removes haemoglobin as rapidly as would an exchange transfusion, the only possible therapeutic procedure.

REFERENCES.

Black, R. H. (1948): Ann. trop. Med. Parasitol., 42, p. 90.
Brading, I., George, E. P., and Walsh, R. J. (1956): Austral. Ann. Med., 5, p. 118.
Fairley, N. H. (1941): Quart. J. Med., 10, p. 95.
Finch, C. A., Thomas, E. D., Walsh, R. J., and Fluharty, R. G. (1948): J. clin. Invest., 27, p. 533.
Jandl, J. H., Richardson Jones, A., and Castle, W. B. (1957): *Ibid.*, 36, p. 1428.
Rather, L. J. (1948): Ann. trop. Med. Parasitol., 42, p. 90.
Walsh, R. J., Arnold, Barbara J., Lancaster, H. O., Coote, Margaret A., and Cotter, Helen (1953): National Health and Medical Research Council. Special Report. Series No. 5. Canberra.
Yuile, C. L. (1942): Physiol. Rev., 22, p. 19.

THE EFFECT OF RECEPTOR DESTROYING ENZYME, TRYPSIN AND PAPAIN ON RED CELL SURVIVAL

by ILSE BRADING, E. P. GEORGE AND R. J. WALSH

(From the New South Wales Red Cross Blood Transfusion Service, Sydney, and the Physics Department, St. Vincent's Hospital, Sydney).

(*Accepted for publication 2nd October, 1958.*)

SUMMARY.

Red cells, treated *in vitro* with papain, trypsin or receptor destroying enzyme had reduced *in vivo* survival times as determined by the Cr^{51} method. The papain-treated cells were destroyed at random at a fairly constant rate. Trypsin produced irreversible changes in the cells and the rate of destruction was greatest shortly after injection. RDE-treated cells were removed for the first four or five days but thereafter survived normally. The changes produced by the enzyme were apparently reversible. RDE solution was injected intravenously and produced panagglutinable changes in the red cells. These altered cells were removed from the circulation by the anti-T agglutinin in the serum and the rate of removal was dependent on the amount of anti-T present.

INTRODUCTION.

French and Ada (1954) showed that the receptor destroying enzyme (RDE), purified from broth cultures of vibrio cholera, acted on the red cells of guinea-pigs *in vivo*. Following injection of RDE the electrophoretic motility of the red cells was reduced. This change was, however, apparently reversible and the cells regained their former motility in about 21 days. These authors were unable to demonstrate destruction of red cells in their experimental animals. On the other hand, Ejby-Poulson (1954 and 1954a) found that two guinea-pigs injected with RDE prepared from cultures of pneumococcus type 19 developed haemolytic anaemia. In one animal the haemoglobin value fell from 84 p.c. to 43 p.c. in three days, and in the other from 99 p.c. to 46 p.c. in seven days. During recovery from the anaemia the reticulocyte counts rose to 17·9 p.c. and 19 p.c. respectively.

Ejby-Poulson's results raise the possibility that enzymes produced during infections may be concerned in the production of acquired haemolytic anaemia. It was therefore decided to investigate more completely the action of RDE on the survival of red cells. In a series of experiments, rabbit red cells were exposed to RDE *in vitro*, and after labelling with Cr^{51} the treated cells were returned to the circulation. Their survival was determined by the rate of disappearance of the Cr^{51} from the circulation. Purified RDE was also injected intravenously and

the action of the enzyme *in vivo* on labelled cells determined. In addition, the effect of *in vitro* treatment of red cells with papain and trypsin was examined. For purposes of comparison the survival time of normal rabbit red cells was determined in nine animals.

Materials and Methods.

Receptor destroying enzyme RDE.

Purified enzyme, kindly supplied by Dr. E. L. French and Dr. G. R. Ada of the Walter and Eliza Hall Institute, Melbourne, was used both in *in vitro* and *in vivo* experiments. For *in vitro* work the preparation was suitably diluted in calcium acetate saline buffer (pH 6·2), mixed with red cells and placed at 37° C. for 45 minutes as described by French and Ada (1954). The action of the enzyme was stopped by the addition of a small quantity of sodium citrate solution and the cells washed with physiological saline. The RDE content of one preparation was not known. In experiments *in vivo* the RDE was dissolved in 1 ml. of 0·1 p.c. calcium chloride solution, diluted with saline and injected into the ear vein of the animals.

Trypsin.

This was used for *in vitro* action on red cells which were labelled and injected into animals. 0·1 gm. of trypsin (BDH), dissolved in 10 ml. N/20 hydrochloric acid, was diluted with nine parts of M/10 phosphate buffer (pH 7·7). One volume of washed packed red cells was incubated with four volumes of the buffered trypsin solution at 37° C. for varying times as shown in the results. The red cells were washed with saline before being labelled with radio-active chromium.

Papain.

Papain (Difco) was used as described by Stratton (1953). 0·1 gm. of papain was dissolved in 10 ml. physiological saline. This was mixed with 10 ml. of M/10 disodium hydrogen phosphate solution and 20 ml. of 0·2 p.c. L cysteine hydrochloride in saline. An equal volume of this solution was added to washed packed cells and the mixture incubated at 37° C. for 10 minutes. The cells were washed with saline before labelling.

Labelling of red cells.

Radio-active sodium chromate solution ($Na_2Cr^{51}O_4$) was obtained from the A.E.R.E., Harwell, through the Commonwealth X-Ray and Radium Laboratories, Melbourne. Red cells were labelled by mixing 5 ml. of washed packed cells with 1·5 ml. of Cr^{51} solution containing approximately 30 μc. The mixture was placed at 37° C. for 45 minutes, during which time the tube was frequently shaken. The labelled cells were washed three times with physiological saline before being injected into the ear vein.

Radio-assay of blood samples.

For radio-assay, 2 ml. of each blood sample were diluted to 10 ml. with distilled water and approximately 0·5 gm. of saponin added. The radioactivity of all samples was measured at the Adolf Basser Radio-Isotope Laboratory at St. Vincent's Hospital.

Serological tests.

The action of RDE on the red cells was tested by mixing on a tile one drop of washed RDE-treated cells in a 2 p.c. saline suspension with one drop of rabbit serum containing anti-T agglutinins. Macroscopic agglutination in 5 minutes indicated that the T agglutinogen had been unmasked.

In some experiments the presence and titre of the anti-T agglutinin was measured in the rabbit's serum. The sera were serially diluted in tubes with physiological saline and

one drop of each dilution mixed on a tile with one drop of a 2 p.c. suspension of RDE-treated rabbit red cells. The mixtures were examined for agglutination after 5 minutes and the titre of the anti-T taken as the last dilution of the serum producing visible agglutination.

The activity of trypsin and papain on red cells was tested by treating human group O Rh-positive cells with the enzymes at the same time as the rabbit's cells were treated. The washed human cell suspension was then mixed with incomplete anti-D serum in a test tube and incubated at 37° C. for 1 hour. Macroscopic agglutination indicated enzymatic activity on the cells. Incomplete anti-D serum does not agglutinate normal Rh-positive red cells suspended in saline, but it does agglutinate cells treated with trypsin or papain (Morton and Pickles, 1947; Stratton, 1953). Neither trypsin nor papain exposed the T agglutinogen.

In some experiments Cr^{51}-labelled red cells were injected intravenously into the rabbits prior to injection of RDE solution. The possibility that the presence of chromium on the red cells might interfere with the action of RDE was investigated *in vitro* by mixing red cells with Na_2CrO_4 solution before treatment with RDE. The chromium solution did not prevent exposure of the T agglutinogen.

RESULTS.

Normal red cell survival.

Samples of venous blood were collected from each of eight rabbits 20 minutes, 2 hours and 18 hours after injection of their own labelled cells. Subsequently, samples were obtained at various intervals for 10 weeks. The radioactivity in the first samples was taken as 100 p.c. and that in the other samples, after correction for decay, was expressed as a percentage of this initial value. The individual values are plotted in Fig. 1 and a curve of best fit is drawn through the points.

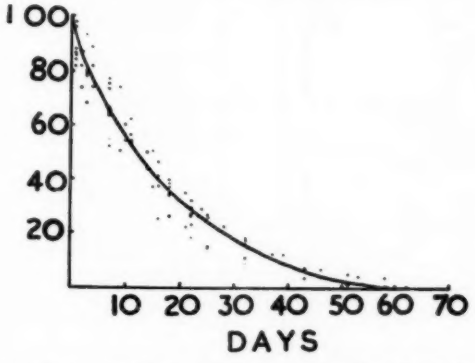


Fig. 1. Red cell survival in rabbits by the Cr^{51}-labelling method. In every instance the radioactivity in the first sample collected 10 mins. after injection of the labelled cells has been taken as 100 p.c. and other measurements related to this value.

It appears from the curve and from studies with Fe^{59} in rabbits of the same species (Clark, 1958) that the mean life time of the rabbits' red cells is approximately 57 days. This figure is similar to that found for the rabbit by other workers (Rodnan, Ebaugh and Spivey Fox, 1957).

The curve is due to two factors (a) elution of Cr^{51} from the cells in the circulation, and (b) removal of labelled red cells due to ageing. No correction has been applied for changes in the total red cell volume or haematocrit value re-

TABLE 1.

Percentage survival of Cr^{51}—tagged red cells after in vitro *treatment with enzymes.*

Enzyme	Days after injection										
	1	2	4	7	9	10	14	15	17	18	23
Papain	75·5			41·7	39·2			18·6			
Papain	87·0			31·0	17·7			9·8			
Trypsin 40 mins.	15·0	4·5	1·9	0·3	0						
Trypsin 40 mins.	24·2	8·5	1·6	0·7	0						
Trypsin 35 mins.	75·0	59·4	46·8			17·5		8·1		8·0	3·4
Trypsin 25 mins.	81·9	73·1	68·0			41·0		32·6		30·3	23·0
Trypsin 20 mins.	73·5	64·5	52·6	44·1		30·2	26·0			19·3	12·5
Trypsin 20 mins.	80·0	74·8	61·5	57·5		45·1	38·9			29·9	21·5
Trypsin 15 mins.	82·5	75·3	63·0			35·2		25·5		20·1	13·2
Trypsin 10 mins.	79·5	82·0	70·2	64·5		48·1					
Trypsin 10 mins.	77·8	76·5	70·6	59·0		46·9	39·5			31·0	14·9
RDE 1/1	38·7	28·8	43·8	37·1		28·5	24·0			20·1	
RDE 1/2	45·4			22·7	20·0			16·0			
RDE 1/2	50·9			31·0	36·8			20·3			
RDE 1/2	30·4	21·0	28·6	22·2		16·2	12·6			9·1	
RDE 1/2	18·5	6·8	26·2	27·3		20·3	21·6			15·3	
RDE 1/4	51·0	38·0	35·8	30·0		23·8	17·5			14·7	
RDE 1/4	22·8	18·7	20·0	15·4		11·0	5·3			6·9	
RDE 80,000 units	24·5	33·0		24·2	24·2		16·2		19·7		
RDE 80,000 units	16·0	45·0		31·5	27·2						

sulting from the removal of the samples. In subsequent work, comparisons of cell survival were made with the curve in Fig. 1.

The rate of elution of Cr^{51} from the normal cells was calculated by dividing the percentage of Cr^{51} for various values of t in Fig. 1 by $1 - {}^{t}/_{57}$ and plotting these values against t on semi-logarithmic paper. From the straight line obtained it was found that half the Cr^{51} was eluted in 21·2 days. The loss of Cr^{51} per day was then calculated from $100\,(1 - \sqrt[21.2]{0{\cdot}5})$ as 3·2 p.c. This is greater than the 1 p.c. which occurs in humans (Mollison, 1956). In all subsequent experiments it has been assumed that the rate of Cr^{51} elution is the same for treated as for untreated rabbit cells. The possibility that the rate may be altered has not been investigated.

In vitro *action of enzymes on red cells.*

Some results of the different experiments measuring the survival of red cells treated *in vitro* with the enzymes are shown in Table 1. The observed values were compared with the survival values obtained for untreated red cells at the same time after injection, the normal values being obtained from Fig. 1. The ratio of Cr^{51} treated cells/Cr^{51} untreated cells eliminates the factors of Cr elution and normal removal of cells due to senescence. Any departure of this ratio from unity represents accelerated destruction due to the experimental procedures. These ratios were plotted logarithmically against time and the line or curve of best fit drawn through the points.

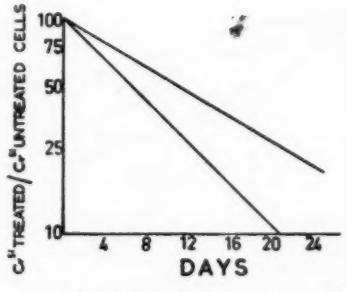


Fig. 2. Rate of destruction of papain treated red cells in two rabbits. In this and the remaining figures the ordinate shows the ratio of the injected Cr^{51} to the Cr^{51} which would have been present after the same time interval if the cells had not been treated (see text).

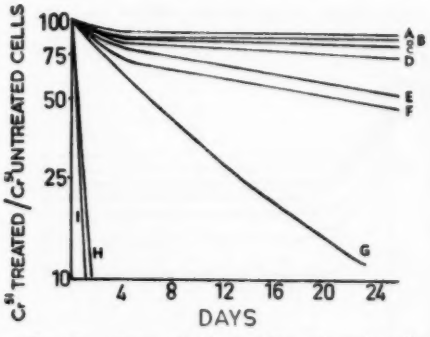


Fig. 3. Rate of destruction of red cells treated with trypsin *in vitro*. The enzyme was allowed to act for the following periods of time: A, 10 mins.; B, 10 mins.; C, 25 mins.; D, 20 mins.; E, 15 mins.; F, 20 mins.; G, 35 mins.; H, 40 mins.; I, 40 mins.

When accelerated destruction occurred, four possible explanations were considered:—

(a) The enzyme action may have altered the viability of all cells but the older cells to a greater extent than the younger cells. This possibility would produce a straight line or a curve convex to the time axis. If the younger cells were affected more than the older, a concave curve would be obtained.

(b) The cells are destroyed at random irrespective of their age. This would produce a straight line.

(c) The cells are removed at random but gradually recover from the action of the enzyme. The curve would first be convex to the time axis and when recovery is complete continue as a line parallel to the time axis.

(d) The enzyme may alter only a percentage of all the red cells and these cells are rapidly removed. A curve similar to (c) would then be obtained.

Papain. The results shown in Fig. 2 are linear, suggesting that the mechanism is either (a) or (b) above. However, random destruction (b) seems a more likely possibility.

Trypsin. The results shown in Fig. 3 for the trypsin-treated red cells show considerable variation. Prolonged action of trypsin (40 minutes) leads to rapid elimination of the cells from the circulation of the recipient, whereas short exposure (10 minutes) is responsible for little deviation from the normal. The curves resulting from intermediate periods of exposure are not entirely consistent in relation to the time of action of the enzyme. In general, the rate of red cell destruction is most rapid in the early stages and then becomes less. The mechanism is probably either (a) or (b) above.

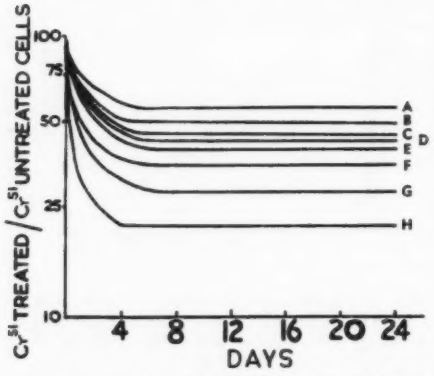


Fig. 4. Rate of destruction of red cells treated with RDE *in vitro*. The enzyme preparation was diluted as follows: A, undiluted; B, 1 in 2; C, 80,000 units; D, 1 in 2; E, 1 in 4; F, 1 in 2; G, 1 in 2; H, 1 in 4.

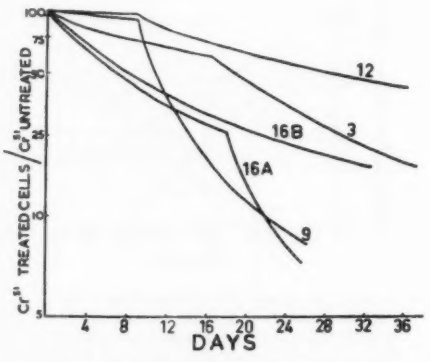


Fig. 5. Rate of destruction of red cells following intravenous injection of RDE solution. The rabbit numbers are referred to in the text.

TABLE 2.

Percentage survival of Cr^{51} tagged red cells in some rabbits injected with RDE solution.

Rabbit No.	Units of RDE injected	Days after injection															
		1	2	3	4	7	9	10	11	14	15	17	18	21	22	28	37
12	60,000	86·2		79·4		66·0	54·0			34·1		28·1			18·4	10·9	5·0
16A	120,000	85·5	78·1		60·0	38·4	27·5		20·8		13·4		9·6	3·5			
16B	120,000	82·5	78·5	69·4		34·5	25·4			16·3		12·0			9·1	40·0	1·4
3	120,000	82·1	75·8	73·5		56·6	42·1			29·6		21·0			13·0	5·0	2·1
9	240,000	82·8	88·0		75·5	60·0		41·5	23·4	12·8			5·3		2·9		

Receptor destroying enzyme (*RDE*). The results for the RDE-treated cells shown in Fig. 4 produce a consistent type of curve but not a consistent relationship between the enzyme concentration and the subsequent fate of the red cells. In different animals a percentage of the treated cells was destroyed in the first four to five days, and the remainder survived normally. It is likely that the cells were removed at random in the early period and that the changes in the cells produced by the enzyme were gradually reversed as discussed above under (c). This is supported by the findings of French and Ada (1954). The possibility mentioned under (d) could also be considered as it would produce the same pattern.

It is of interest that in two rabbits the anti-T agglutinin was reduced in titre by the injection of the treated cells but increased in titre after 9 days. This raised the possibility that the removal of the treated cells in the early stages is related to the action of this anti-T agglutinin. Support for this was obtained from the *in vivo* experiments with RDE.

In vivo *action of RDE.*

Four rabbits were injected with approximately 5 ml. of their own red cells which had been tagged with Cr^{51}. After a few minutes RDE solution was injected into the vein of the opposite ear. The survival of the tagged cells was measured, and some of the results are shown in Table 2. The calculated ratios of the observed to the normal survivals are plotted in Fig. 5. One rabbit (No. 16) was used on two occasions. Another (No. 9) was injected on the 9th day with 14 ml. of plasma from another rabbit. This plasma contained anti-T agglutinins but no antibody active against the recipient's untreated red cells. In all rabbits the red cells were tested at frequent intervals, for exposure of the T-antigen, and the sera were examined for the presence of anti-T.

In four of the five tests the curves shown in Fig. 5 appear to have two components, the exception being the second occasion on which Rabbit No. 16 was used (16B). In two animals (Nos. 9 and 12) there is little deviation from the normal rate of destruction during the first nine days; whilst the other animals show slow destruction of red cells for 16 to 18 days. The second components represent more rapid rates of destruction. In Rabbit No. 9 this second component commenced immediately following the injection of the serum containing anti-T.

Changes in the T-antigen of the red cells and of the anti-T agglutinin content of the serum are shown in Table 3. Following injection of the enzyme the red cells of three animals were rendered panagglutinable for varying periods, but towards the later part of the experiment the T-antigen could not be detected. In one animal (Rabbit No. 12) the T-antigen was not demonstrable at any time. With the exception of this same animal, anti-T was not found in the sera for some days following the RDE injections, but reappeared later in every instance.

Changes in the haemoglobin values and reticulocyte percentages of three animals which received RDE intravenously are shown in Table 4. The haemoglobin values fell by 4·7, 3·0 and 5·2 gm. and these changes were associated with increases in the reticulocytes to 9·4 p.c., 10·1 p.c. and 13·7 p.c. respectively. Haemolytic anaemia was therefore produced in rabbits in the same manner as it was produced by Ejby-Poulson (1954a) in guinea-pigs.

TABLE 3.

Changes in the T antigen and in the anti-T content of the serum following injection of receptor destroying enzyme (RDE).

Rabbit No.	T antigen exposed	T antigen NOT exposed	anti-T in serum	anti-T NOT in serum
9	1 to 29 days	0, 39+ days	0, 18+ days	1 to 14 days
12	—	0 to 37 days	0 to 37 days	—
3	1 to 17 days	0, 22+ days	0, 28+ days	1 to 22 days
16B	1 to 7 days	0, 17+ days	0, 7+ days	1 to 3 days

TABLE 4.

Haemoglobin values and reticulocyte percentages of three rabbits injected with RDE.

Time after injection	Rabbit No. 3		Rabbit No. 12		Rabbit No. 16B	
	Hb (gm.)	Reticulocyte %	Hb (gm.)	Reticulocyte %	Hb (gm.)	Reticulocyte %
10 mins.	16·1	2·6	13·6	1·3	12·35	2·2
1 day	14·45		12·1		10·8	
2 days	13·8		12·6		11·25	
3 days	14·0		11·8		10·6	
7 days	13·9	2·9	11·8	0·3	7·6	7·4
9 days	12·1	3·4	11·4	7·0	7·15	13·7
14 days	11·4	4·2	10·6	10·1	8·6	7·0
17 days	12·75	9·4	11·3	5·2	8·9	3·4
22 days	13·9	6·5	11·25	1·7	9·7	2·7
28 days	13·4		11·7		10·7	
37 days	14·75		11·9		10·9	

DISCUSSION.

The results clearly show that when rabbit red cells are treated *in vitro* with RDE, trypsin or papain, the survival time in the circulation is reduced. Papain and trypsin appear to produce irreversible changes in the cells and these changes themselves lead to destruction of the cells. On the other hand, the changes produced by RDE are reversible and do not of themselves lead to removal of the cells. The union of the anti-T agglutinin in the serum with the exposed T agglutinogen of the red cells seems necessary before the red cells are destroyed.

The enzymes used in the work would be expected to act on the surface of

the red cells rather than on the protein compounds present within the cell. RDE attacks the neuraminic acid (Gottschalk, 1957) whilst the proteolytic enzymes, papain, trypsin, would act on the protein moiety of the lipo-protein complex comprising the red cell membrane. Ponder (1948) points out that if allowed to act for a sufficient time papain will produce haemolysis *in vitro*. Intracellular enzyme systems appear to play some part in determining the life-time of the red cell. Gabrio, Finch and Huennekens (1956) have shown that resynthesis of ATP from adenosine by way of a nucleoside phosphorylase system is important in the preservation of red cells during storage. Szeinberg, Sheba and Adam (1958) demonstrated that the glutathione — reduced glutathione system is deficient in paroxysmal nocturnal haemoglobinuria and in haemolytic anaemias due to drugs. The results in this paper raise the possibility that changes in the red cell surface due to proteolytic enzymes may also determine survival of red cells.

The changes produced by intravenous injection of RDE, (1) in the survival of the labelled cells, (2) in the exposure of the T antigen of the red cells, and (3) in the anti-T content of the serum, can be correlated by postulating the following sequence of events:

(a) Following injection of the enzyme the T antigen of the red cells is unmasked, although this could not be demonstrated in one instance (Rabbit No. 12) by the insensitive methods employed.

(b) The unmasked T antigen of the cells absorbs the anti-T agglutinin present in the serum leading to destruction of some of the cells and disappearance of anti-T from the serum. This obviously occurred in Rabbit No. 9, following injection of plasma containing anti-T.

(c) Injection of RDE increases, directly or indirectly, the anti-T content of the serum (French and Ada, 1954), and this immune anti-T produces an increased rate of red cell destruction—the second component seen in Fig. 5. At a later stage the excess of anti-T is found free in the serum.

Experimental haemolytic anaemia has been produced by injections of RDE in both rabbits and guinea-pigs, but not as a continuing disease in either species. It does not seem likely, however, that changes in red cells produced by RDE are involved in the production of acquired haemolytic anaemia in man. Amongst other things, antibody eluted from coated red cells in this condition acts on unaltered red cells; anti-T does not do this. Nevertheless, the possibility cannot be entirely discounted because panagglutination *in vivo*, described as polyagglutinability, has been reported by a number of workers (Basil-Jones, Sanger and Walsh, 1946; Boorman, Loutit and Steabben, 1946; Gaffney and Sachs, 1943).

REFERENCES.

Basil-Jones, B., Sanger, R. A., and Walsh, R. J. (1946): Nature, **157**, p. 802.
Boorman, K. E., Loutit, J. F., and Steabben, D. B. (1946): *Ibid.*, **158**, p. 446.
Clark, P. (1958): Personal communication.

Ejby-Poulsen, P. (1954): Nature, 173, p. 82.
Ejby-Poulsen, P. (1954a): *Ibid.*, 174, p. 929.
French, E. L., and Ada, G. L. (1954): Austral. J. exp. Biol., 32, p. 165.
Gabrio, B. W., Finch, C. A., and Huennekens, F. M. (1956): Blood, 11, p. 103.
Gaffney, J. C., and Sachs, H. (1943): J. Path. Bact., 55, p. 489.
Gottschalk, A. (1957): Physiol. Rev., 37, p. 66.
Mollison, P. L. (1956): Blood Transfusion in Clinical Medicine. 2nd ed. Blackwell Scientific Publications, Oxford. Chapter: Normal life span of red cells, p. 133.
Morton, J. A., and Pickles, M. M. (1947): Nature, 159, p. 779.
Ponder, E. (1948): Hemolysis and Related Phenomena. Grune and Stratton, New York, p. 139.
Rodnan, G. P., Ebaugh, F. G., Jr., and Spivey Fox, M. R. (1957): Blood, 12, p. 355.
Stratton, F. (1953): Lancet, 1, p. 1169.
Szeinberg, A., Sheba, C. H., and Adam, A. (1958): Nature, 181, p. 1256.

FLAVINS OF *LACTOBACILLUS ARABINOSUS* 17.5

A LACTIC DEHYDROGENASE CONTAINING A FLAVIN PROSTHETIC GROUP

by A. M. SNOSWELL[1]

(From the Department of Biochemistry, University of Adelaide).

(*Accepted for publication 20th October, 1958.*)

SUMMARY.

The flavin content of *Lactobacillus arabinosus* 17.5 was found to increase by approximately 20 p.c. when the cells were grown under anaerobic conditions, compared with aerobic conditions.

The enzymes flavokinase and adenyl-transferase, involved in the synthesis of FMN and FAD respectively, also showed increased specific activities under these conditions.

The flavoprotein enzymes lactic dehydrogenase and DPNH-menadione reductase also showed increased activity under anaerobic growth while the non-flavoprotein enzymes, pyruvic reductase and 3-phosphoglyceraldehyde dehydrogenase, showed very little variation.

The lactic dehydrogenase was purified some 20-fold from cell-free extracts and was shown to be free of pyruvic reductase. This enzyme was DPN-independent and stoichiometrically converted L-lactate to pyruvate in the presence of 2,6 dichlorophenol-indophenol.

This enzyme would only react with two electron acceptors and not with molecular oxygen.

The anti-malarial substances atabrine and quinine sulphate inhibited enzyme activity. Atabrine inhibition was reversed by the addition of FMN and FAD (FMN being more effective).

The absorption spectrum was fairly typical of a flavoprotein and the flavin peak at 450 mμ was specifically reduced by L-lactate. Approximately 30 p.c. of the total reducible material was reduced by the lactate.

The role of flavoproteins, in general, and specifically of the lactic dehydrogenase, in the metabolism of *Lactobacillus arabinosus* has been discussed.

INTRODUCTION.

Lactobacillus arabinosus 17.5 is a micro-aerophilic organism and indeed shows increased growth in the complete absence of oxygen. This organism contains considerable amounts of flavin (Snoswell, 1957, and DeLuca *et al.*, 1956). Also, no detectable amounts of cytochromes can be found in this organism (Strittmatter, 1958; cf. text). These facts suggest that flavins may play an important role in oxidation reactions in *L. arabinosus*.

---

[1] This work was supported by a National Health and Medical Research Council grant.

The present paper indicates that not only does this organism contain more flavin when grown under anaerobic compared with aerobic conditions, but also the enzymes responsible for the synthesis of FMN[2] and FAD (flavokinase and adenyl-transferase respectively) show an increased specific activity under anaerobic conditions.

During investigations on the metabolism of *L. arabinosus* a rapid oxidation of lactic acid was observed with cell-free extracts under suitable conditions. Whereas numerous cases of the oxidation of lactic acid by micro-organisms have been reported (Yamamura *et al.*, 1952; Appleby and Morton, 1954; Edson, 1954; Cousins, 1956; Sutton, 1957) such an oxidation by a lactic acid-producing organism is rather unusual. Hager, Geller and Lipmann (1954) have reported a lactic oxidase in *L. delbrueckii*, but little information is given concerning this enzyme.

Preliminary investigations of the lactic acid oxidation by *Lactobacillus arabinosus* suggested that this reaction was not merely a reversal of the normal DPN-dependent lactic dehydrogenase reaction, but was catalysed by an entirely different enzyme, which was DPN-independent. As the equilibrium of the DPN-dependent reaction lies largely in the direction of pyruvate reduction, the enzyme catalysing this reaction is referred to as pyruvic reductase in this paper.

The present paper also describes the partial purification and properties of the DPN-independent lactic dehydrogenase which was shown to be a flavoprotein enzyme.

Methods and Materials.

Lactobacillus arabinosus 17.5 was grown on media previously described (Snoswell, 1957).

Anaerobic cultures of the organism were grown in Florence flasks, of 8 litre capacity, which were continually agitated by a stream of gas containing 95 p.c. nitrogen and 5 p.c. carbon dioxide. Traces of oxygen were removed from this gas by passage through a heated copper tube, and then through a trap containing 10 p.c. sodium hydrosulphite, and finally through a distilled water trap. The cells used for inoculation were passed through three successive sub-cultures under these strict anaerobic conditions.

Aerobic cultures of *Lactobacillus arabinosus* were grown under similar conditions but using a stream of gas, containing 95 p.c. oxygen and 5 p.c. carbon dioxide, for aeration.

Cell-free extracts of the organism were also prepared as described previously (Snoswell, 1957).

Reagents. Riboflavin, DPN (75 p.c.), FMN and FAD (90 p.c.) were obtained from Sigma Biochemical Co. Fumaric acid, sodium pyruvate, sodium nitrate, D-L lithium lactate, quinine sulphate and 2,6 dichlorophenol-indophenol were laboratory reagent chemicals (British Drug Houses Ltd.). Atabrine (as Mepacrine Hydrochloride) was kindly supplied by Imperial Chemical (Pharmaceuticals) Ltd. Lactic acid dehydrogenase (twice crystallised) was supplied by the Nutritional Biochemical Corporation. Janus Green was supplied by Grübler and Co.

---

[2] The following abbreviations have been used in this paper: FMN: flavin mononucleotide (riboflavin 5′ phosphate); FAD: flavin adenine dinucleotide; DPN: diphosphopyridine nucleotide; DPNH: reduced diphosphopyridine nucleotide.

Acetone used in the enzyme fractionation procedures was refluxed with potassium permanganate for 3 hours and then distilled. After drying over calcium chloride the acetone was redistilled.

Estimations. Riboflavin, FMN and FAD were estimated fluorometrically by the method of Burch, Bessey and Lowry (1948) using a Farrand Fluorometer.

Protein was estimated by the biuret method (Robinson and Hogden, 1940). Pyruvate was estimated by measuring the oxidation of DPNH spectrophotometrically, in the presence of crystalline lactic dehydrogenase. Lactate was estimated colorometrically by the method of Barker and Summerson (1941).

Estimation of enzymic activities. Flavokinase and adenyl-transferase were assayed according to the methods described previously (Snoswell, 1957).

Pyruvic reductase, diaphorase, DPNH-menadione reductase and glyceraldehyde-3-phosphate dehydrogenase were assayed spectrophotometrically, using a Beckman spectrophotometer (Model DU), by measuring the rate of oxidation of DPNH at 340 mμ or the rate of reduction of 2,6 dichlorophenol-indophenol at 600 mμ. Pyruvic reductase was assayed according to the method of Kornberg (1955), diaphorase according to the method of Edelhoch *et al.* (1952) and DPNH-menadione reductase according to the method of Wosilait and Nason (1954). In each case a unit of activity was defined as that amount causing a change in optical density of 0·010 per minute.

The glyceraldehyde-3-phosphate dehydrogenase reaction was linked to the diaphorase reaction in order to estimate glyceraldehyde-3-phosphate dehydrogenase activity, as the normal method of assay proved unsatisfactory. The assay system contained: 75 μ moles Na/K phosphate buffer (pH 6·0), 10 μ moles $MgCl_2$, 0·75 μ moles DPN, 0·12 μ moles 2,6 dichlorophenol-indophenol, 10 μ moles glyceraldehyde 3-phosphate and enzyme, in a total volume of 3 ml. Water replaced the substrate in the control cuvette. Reduction of the indophenol was measured at 600 mμ and a unit of enzyme activity was defined as that amount causing a reduction in optical density of 0·010 per minute.

Fumarate reductase was estimated by a modification of the method described by Martin and Morton (1956a). The assay system contained: 100 μ moles Na/K phosphate buffer (pH 7·0), 80 μ moles sodium fumarate, 0·25 μ moles Janus Green, 4·8 μ moles sodium hydrosulphite and enzyme, in a total volume of 2 ml. The substrate, buffer, dye and enzyme were mixed in the main compartment of a Thunberg tube. After evacuation the sodium hydrosulphite (in phosphate buffer) was tipped from the side arm and the time for the complete reoxidation of the reduced dye to safranin was measured. As sodium hydrosulphite is particularly unstable in the presence of oxygen, this solution was prepared fresh, immediately before use and was the last addition to the Thunberg tube before evacuation. Using these precautions good agreement was obtained on repeated assays. One unit of activity was defined as that amount of enzyme causing the reoxidation of 1 mμ. moles of reduced dye per hour and specific activity as units per milligram of protein.

Nitrate reductase was measured in an identical system, except that sodium fumarate was replaced by 80 μ moles sodium nitrate. A unit of activity was defined in a manner similar to the fumarate reductase unit.

Assay of the DPN-independent lactic dehydrogenase. This enzyme was estimated spectrophotometrically in a Unicam SP 500 Spectrophotometer, by measuring the reduction of 2,6 dichlorophenol-indophenol in the presence of lactate. The assay procedure was as follows: To a cuvette of I cm. light path and 3 ml. capacity, the following reagents were added: 100 μ moles DL-lithium lactate, 75 μ moles mixed phosphate buffer (pH 6·0), 0·12 μ moles 2,6 dichlorophenol-indophenol and distilled water to give a final volume of 2·9 ml. Water replaced lactate in the control cuvette. The reaction was started by the addition of 0·1 ml. enzyme solution to each cuvette. Optical density was measured at a wave length of 600 mμ, at 30 sec. intervals, for 3 minutes. One unit of enzyme activity was de-

fined as that amount causing a reduction in optical density of 0·010 in 1 minute and specific activity as units per milligram of protein.

RESULTS.

The flavin content of Lactobacillus arabinosus *grown under aerobic and anaerobic conditions.*

Extracts of *Lactobacillus arabinosus*, prepared by the disintegration of cell suspensions with glass beads, in a high speed shaker (Nossal, 1953), contained 96-100 p.c. of the flavin extractable from whole cells with 10 p.c. trichloro-acetic acid. As cell-free extracts were used for most enzyme studies, the flavin content of these extracts was used to give a measure of total flavin.

When *Lactobacillus arabinosus* was grown under anaerobic conditions there was an approximate 20 p.c. increase in the total flavin content, compared with cells grown under aerobic conditions (Table 1). There was a slightly greater increase in FMN content compared with FAD content under the anaerobic conditions. Total cell production was also stimulated under these conditions.

TABLE 1.

The flavin content of Lactobacillus arabinosus *grown under aerobic and anaerobic conditions.*

Growth condition	Total wt. of cells (gm.)	Results in mμ mole/gm. dry wt. cells			
		Riboflavin	FMN	FAD	Total flavin
Aerobic	5·6	nil	68	216	284
Anaerobic	6·9	nil	87	255	342

Flavin assays were carried out on 0·5 ml. aliquots of cell-free extracts containing 20·2 and 19·4 mg./ml. protein respectively. The figures obtained were converted to mμ mole/gm. dry wt. from the dry wt. content of the cell suspension used in preparing the extract, assuming that all the flavin was released on cell disintegration (see text.)

The biosynthesis of FMN and FAD by Lactobacillus arabinosus *grown under aerobic and anaerobic conditions.*

As the amounts of FMN and FAD in *Lactobacillus arabinosus* were found to increase when cells were grown under anaerobic conditions, it appeared likely that the enzymes, catalysing the synthesis of these flavin nucleotides, may also have been stimulated under these conditions.

Examination of cell-free extracts for flavokinase and adenyl-transferase (previously shown to be involved in the synthesis of FMN and FAD respectively in the organism (Snoswell, 1957)), revealed that the specific activity of both these enzymes showed an appreciable increase in the anaerobic extract compared with the aerobic extract (Table 2).

TABLE 2.

The flavokinase and adenyl-transferase activities of cells grown under aerobic and anaerobic conditions.

Growth condition	Flavokinase (Specific activity)	Adenyl-transferase (Specific activity)
Aerobic	0·24	0·23
Anaerobic	0·38	0·30

Flavokinase was assayed on cell-free extracts containing 20·2 and 19·4 mg./ml. protein respectively. The assay mixture contained 2 μ mole. ATP, 1·5 μ mole. $MgCl_2$, 75 μ mole. Tris buffer (pH 7·0), 10 mμ mole. riboflavin and 0·5 ml. cell-free extract in a total volume of 2 ml. Incubated at 30° C for 1 hr. The increase in FMN over the control was measured fluorometrically.

Adenyl-transferase was assayed similarly with 10 mμ mole. FMN in place of the riboflavin used in the assay mixture. The increase in FAD over the control was measured fluorometrically.

The response of various enzymes to anaerobic growth conditions.

Cells of *Lactobacillus arabinosus* have been shown to contain increased amounts of FMN and FAD when grown under anaerobic conditions (Table 1) and the enzymes involved in the synthesis of these nucleotides showed increased activities under these conditions (Table 2). Thus it was of interest to examine

TABLE 3.

Enzyme activities of cells grown under aerobic and anaerobic conditions.

Growth condition	Lactic dehydrogenase	Diaphorase	DPNH-menadione reductase	Pyruvic reductase	Glyceraldehyde-3-phosphate dehydrogenase
Aerobic	10	102	13	144	0·85
Anaerobic	17	72	21	153	0·82

All figures expressed as specific activities.

Lactic dehydrogenase assay system contained, 100 μ mole. DL-lithium lactate, 75 μ mole. Na/K phosphate buffer (pH 6·0), 0·12 μ mole. indophenol and 0·1 ml. of a 1/10 dilution of cell-free extract (as in Table 2) in a volume of 3 ml. Decrease in optical density at 600 mμ, over the control, was recorded every 30 sec.

Diaphorase assay system contained, 150 μ mole. Na/K phosphate buffer (pH 7·5), 0·3 μ mole. DPNH, 0·12 μ mole. indophenol and 0·1 ml. of a 1/100 dilution of cell-free extract (as in Table 1) in a total volume of 3 ml. Decrease in optical density at 600 mμ, over the control, was recorded every 30 sec.

DPNH-menadione reductase assay system contained, 200 μ mole. Na pyro-phosphate buffer (pH 8·4), 0·2 μ mole. DPNH, 0·3 μ mole. menadione, and 0·1 ml. cell-free extract (as in Table 1) in a total volume of 3 ml. Decrease in optical density at 340 mμ, over the control, was recorded every 30 sec.

Pyruvic reductase assay system contained, 75 μ mole. Na/K phosphate buffer (p H7·5), 1 μ mole. Na pyruvate, 0·2 μ mole. DPNH and 0·1 ml. of a 1/100 dilution of cell-free extract (as in Table 1) in a total volume of 3 ml. Decrease in optical density at 340 mμ, over the control, was recorded every 30 sec.

3-Phosphoglyceraldehyde dehydrogenase assay system contained 75 μ mole. Na/K phosphate buffer (pH 6·0), 10 μ mole. $MgCl_2$, 0·75 μ mole. DPN, 0·12 μ mole. indophenol, 10 μ mole. glucose -6-PO_4 and 0·1 ml. cell-free extract (as in Table 2) in a total volume of 3 ml. Decrease in optical density at 600 mμ, over the control, was recorded every 30 sec.

the activities of suspected flavoprotein enzymes compared with non-flavoprotein enzymes of cells grown under anaerobic and aerobic conditions.

Cell-free extracts were thus examined for lactic dehydrogenase, DPNH-menadione reductase and diaphorase activities and compared with pyruvic reductase and glyceraldehyde-3-phosphate dehydrogenase activities. Table 3 shows the results of this enzyme survey and indicates the activities of the lactic dehydrogenase and DPNH-menadione reductase increased under anaerobic conditions, whereas diaphorase activity fell slightly and pyruvic reductase and glyceraldehyde-3-phosphate dehydrogenase activities showed virtually no alteration.

Fractionation and purification of the lactic dehydrogenase.

Preliminary experiments indicated that the enzyme was heat labile, for 5 min. incubation at 60° C. caused a destruction of 60 p.c. of the activity. This destruction was not protected by the presence of lactate. Thus any heating procedures were avoided. Also the enzyme lost considerable activity when the pH was lowered below 5. Ammonium sulphate fractionation resulted in considerable losses of activity, possibly due to release of the prosthetic group. Finally, a procedure involving acetone fractionation followed by calcium phosphate gel treatment was adopted. However, the ionic strength at which the acetone fractionation was carried out was found to be extremely critical. Ionic strengths of 0·04 μ and 0·06 μ were used, but it was found essential for satisfactory separation, that half this ionic strength should be made up by magnesium acetate. The procedure indicated below was finally adopted.

The cell-free extract which had been prepared in 0·1M Na/K phosphate buffer, was dialysed against distilled water overnight, which reduced the ionic strength to 0·02 μ (determined from inorganic phosphate estimations). Magnesium acetate (1·43 mg./ml.) was added to give a final ionic strength of 0·04 μ. Purified acetone was then added from a dropping funnel to the extract, which was stirred constantly with the aid of a magnetic stirrer. Acetone was added to give a final conc. of 30 p.c. During the acetone fractionations the temperature was maintained at $-10°$ C. by the use of an alcohol bath. After 15 min. equilibration the precipitate was removed by centrifuging at 6000 x g. in a Servall refrigerated centrifuge for 15 min. The supernatant was then brought to 43 p.c. concentration by the addition of more acetone and the process repeated. The acetone concentration was finally raised to 55 p.c. and the 43-55 p.c. acetone fraction dissolved in ice-cold 0·005M Na/K phosphate buffer (pH 7·0) and then freeze-dried to remove acetone.

The freeze-dried fraction was dissolved in distilled water and made up to the original volume. Magnesium acetate (2·14 mg./ml.) was added to raise the ionic strength from 0·03 μ to 0·06 μ and the acetone fractionation repeated. At this second acetone fractionation the 36-40 p.c. fraction contained the majority of the activity.

TABLE 4.

A summary of the purification of the lactic dehydrogenase.

Fraction	Total volume ml.	Units/ml.	Total units	Protein mg/ml.	Specific activity units/mg.	Recovery p.c.	FMN protein	Pyruvic reductase specific activity
Cell-free extract	114	100	11,400	10·8	9·3	—	0·15	144
43-55 p.c. acetone fraction (at 0·04 μ ionic strength)	12·4	462	5,740	8·1	57	50	0·32	47
36-40 p.c. acetone fraction (at 0·06 μ ionic strength)	1·9	1,470	2,750	7·8	189	24	0·45	2·8
Supernatant after calcium phosphate gel treatment.	1·9	1,380	2,620	7·4	186	23	0·46	nil

Lactic dehydrogenase was assayed as in Table 3. Pyruvic reductase also was assayed as in Table 3. FMN was measured by differential fluorometric assay.

After freeze drying, the second acetone fraction was again dissolved in distilled water to the original volume and the pH lowered to 5·7 by the addition of 0·2M acetic acid. Calcium phosphate gel was added (0·2 mg./mg. protein) and after 15 min. equilibration the gel was removed by centrifugation. The supernatant contained the lactic dehydrogenase activity.

Table 4 gives a summary of a typical fractionation. This table also shows the concurrent decrease in pyruvic reductase activity, the final fraction being completely free of this major contaminating enzyme. The calcium phosphate gel treatment, although not increasing the specific activity of the lactic dehydrogenase, was retained to remove the pyruvic reductase, which could be subsequently eluted from the gel with 0·01M Na/K phosphate buffer at pH 8·0.

Properties of the lactic dehydrogenase.

Stability. The final fraction retained 50 p.c. of its enzymic activity after storage at − 15° C. for two months.

Rate studies. Enzyme activity followed a zero order reaction for 1½ hr. (Fig. 1). Half maximal velocity was observed when the concentration of L-lactate was $1 \cdot 1 \times 10^{-2}$M (K_m value) or $2 \cdot 2 \times 10^{-2}$M for DL-lactate as determined from the Lineweaver Burk Plot (Fig. 2). Optimal enzyme activity was observed at pH 6·0 (Fig. 3). Thus the enzyme was specific for L-lactate. There was no reaction using sodium glycolate as a substrate.

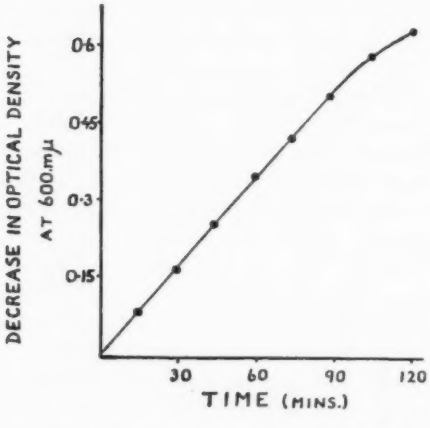


Fig. 1. *The rate of the lactic dehydrogenase reaction.* The assay system contained 100 μ moles DL-lithium lactate, 75 μ moles Na/K phosphate buffer (pH 6·0), 0·24 μ moles indophenol, and 0·005 mg. enzyme in a total volume of 3 ml. Decrease in optical density, over the control, was measured at the time intervals indicated in the figure.

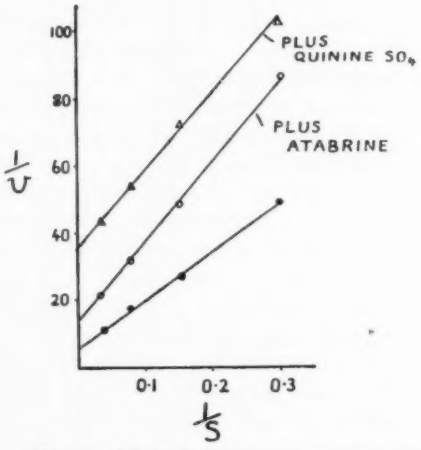


Fig. 2. *Effect of lactate concentration and inhibitors on enzyme activity.* (Lineweaver Burk plot) Ordinate 1/v where v = units of activity. Abscissa, 1/S where s = conc. of DL-lithium lactate. Standard assay conditions were used, as in Table 3. 0·005 mg. of enzyme was used per assay.

Reaction products and stoichiometry. The oxidation of lactic acid, by some micro-organisms, has been shown to be accompanied by decarboxylation, e.g. the lactic oxidative decarboxylase of *Mycobacterium phlei*, degrades L-lactic acid to acetic acid and carbon dioxide (Sutton, 1957). However, no liberation of carbon dioxide was observed manometrically during the oxidation of lactate by the lactic dehydrogenase of *Lactobacillus arabinosus*. Thus it was likely that the reaction involved a simple dehydrogenation of lactate to pyruvate.

Pyruvic acid was identified as a reaction product by the use of crystalline rabbit muscle lactic dehydrogenase (or pyruvic reductase) which reduces pyruvate to lactate in the presence of DPNH. The amount of DPNH oxidized gave a measure of pyruvate present (β keto derivatives of short chain fatty acids are also reduced by this enzyme, but these compounds would not have been present in this case).

TABLE 5.

Stoichiometry of the lactic dehydrogenase reaction.

Lactate oxidized	Indophenol reduced	Pyruvate reduced
5·6 μ mole.	5·4 μ mole.	5·4 μ mole.

Incubation mixture: 125 μ mole. Na/K phosphate buffer (pH6·0), 10 μ mole. L-lithium lactate, 5·4 μ mole. indophenol and 2 mg. enzyme in a total volume of 4 ml. Incubation at 30°C. Completion of the reaction was taken when all indophenol reduced (45 min.) Aliquots were immediately removed for lactate and pyruvate assays.

Using the 20-fold purified lactic dehydrogenase, a stoichiometric balance between the amount of lactate oxidized, 2,6 dichlorophenol-indophenol reduced and pyruvate produced, was demonstrated (Table 5). In the presence of high concentrations of reduced indophenol and pyruvate, some re-oxidation of the indophenol did occur, indicating the lactic dehydrogenase would catalyse the reverse reaction. However, the high redox potential of the indophenol compared with the pyruvate-lactate couple (E_o^1 + 0·127v. against 0·00v. at pH 7·0) makes such a reverse reaction unfavourable, but, even using reduced Janus Green (E_o^1 at pH 7·0 of −0·225v.) only a very slow oxidation of dye occurred. Thus, although an equilibrium constant was not actually determined for the lactic dehydrogenase reaction, it appeared that the equilibrium lay predominantly in the direction of lactate oxidation.

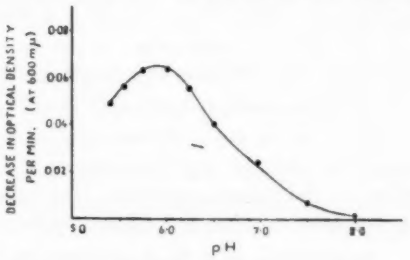


Fig. 3. *Effect of pH on enzyme activity.* Standard assay conditions were used, as in Table 3, except Na/K phosphate buffer of the pH indicated in the figure was used in the pH range 6·0-8·0 and acetate buffer below pH 6·0. pH values below 5·4 could not be used as the indophenol dye changed colour at this level. 0·005 mg. of enzyme was used per assay.

Inhibitor studies. Both atabrine (final conc. 3 x 10^{-4}M) and quinine sulphate (final conc. 5 x 10^{-5}M) acted as non-competitive inhibitors of the lactic dehydrogenase (Fig. 3). Haas (1944) demonstrated that quinine sulphate and atabrine were specific inhibitors of flavoprotein enzymes. The inhibition produced by various concentrations of these two compounds is shown in Table 6. This table indicates that, at the same concentration, quinine sulphate is more active as an inhibitor than atabrine. A similar observation was noted by Eichel (1956) in studying the inhibition of succinate oxidation by *Tetrahymena pyriformis* in the presence of these two compounds.

TABLE 6.

Inhibition of lactic dehydrogenase activity.

Final conc. of inhibitor	p.c. Inhibition with atabrine	p.c. Inhibition with quinine SO_4
3×10^{-6}M	NIL	12
3×10^{-5}M	11	57
1×10^{-4}M	24	78
3×10^{-4}M	62	93
6×10^{-4}M	72	100

Lactic dehydrogenase was assayed as in Table 3. The assay system, included concentrations of inhibitor given in the Table.

The inhibition produced by atabrine was reversed by the addition of FMN or FAD (Table 7). However, the addition of these flavin nucleotides produced little effect on the quinine sulphate inhibition. Again, this was similar to the results found by Eichel (1956) with the succinoxidase system of *Tetrahymena pyriformis*. Table 7 also shows that FMN was slightly more effective than FAD (at the same concentration) in reversing the atabrine inhibition. It is possible, therefore, that FMN is the prosthetic group of the enzyme.

p-Chloromercury-benzoate at a final concentration of $1{\cdot}7 \times 10^{-4}$M had no effect on the lactic dehydrogenase reaction. Also, DPN at a final concentration of 3×10^{-4}M and pyruvate at a final concentration of 3×10^{-2}M had no effect on the enzyme reaction. The latter observation is similar to that observed by Appleby and Morton (1954) with yeast lactic dehydrogenase.

Menadione at a final concentration of $1{\cdot}0 \times 10^{-4}$M inhibited the reaction by 25 p.c., possibly suggesting that menadione could act as an alternative electron acceptor.

Electron acceptors. Of the electron acceptors tested in the lactic dehydrogenase reaction, only two electron acceptors were found effective. 2,6 dichlorophenol-indophenol (as used in the enzyme assay) was most effective, although 1-napthol-2-sodium sulphonate indo-2,6 dibromophenol (E_o^1 at pH $7{\cdot}0 + 0{\cdot}119$v.) and Bindschedler's Green (E_o^1 at pH $7{\cdot}0 + 0{\cdot}224$v.) could act as electron

TABLE 7.

Reversal of the inhibition of lactic dehydrogenase activity.

Final conc. of flavin nucleotide	p.c. Reversal of atabrine inhibition	p.c. Reversal of quinine SO_4 inhibition
3×10^{-6}M FMN	14	5
3×10^{-6}M FAD	8	2
3×10^{-5}M FMN	100	16
3×10^{-5}M FAD	93	4

Lactic dehydrogenase was assayed as in Table 3. The assay system included atabrine at a final conc. of 3×10^{-4}M (giving 62 p.c. inhibition) or quinine sulphate at a final conc. of 3×10^{-5}M (giving 57 p.c. inhibition). The concentrations of flavin nucleotides indicated in the Table were also included in the assay system.

acceptors, but less efficiently. The enzyme did not catalyze the reduction of ferricyanide, cytochrome C or molecular oxygen (no oxygen uptake was observed manometrically, either in the presence or absence of catalase). However, oxygen uptakes could be demonstrated, using freshly prepared cell-free extracts of *Lactobacillus arabinosus*, with lactate as substrate.

Cell-free extracts of *Lactobacillus arabinosus* contain fumarate and nitrate reductase. However, no reduction of nitrate or fumarate was observed (as measured by nitrite or succinate formation) using lactate as a substrate in the presence of cell-free extracts of this organism.

The nature of the prosthetic group.

Results presented in previous sections of this paper and tabulated below, together, suggested that the lactic dehydrogenase of *Lactobacillus arabinosus* contains a flavin prosthetic group.

1. The final preparation of lactic dehydrogenase was free from pyruvic hydrogenase activity (see Table 4) and added DPN had no effect on the lactic dehydrogenase reaction.

2. During the purification of the enzyme an increase in the FMN/protein was observed at each stage (Table 4), whereas the FAD/protein ratio increased at the first stage of purification and subsequently decreased (this latter result was not incorporated into Table 4).

3. The inhibition of the lactic dehydrogenase by the anti-malarial substance atabrine, was reversed by FAD and FMN, the latter compound completely reversing the inhibition.

4. The reduction of indophenol proceeded directly in the absence of DPN.

Determination of the absorption spectrum of this enzyme gave a spectrum similar to that of flavoprotein enzymes. Absorption peaks were found at 275

mμ and 450 mμ with a shoulder around 350 mμ. The absorption peak at 450 mμ was reduced on the addition of lactate and this reduction was about 30 p.c. of that produced by the addition of dithionite (sodium hydrosulphite) as shown in Fig. 4.

The enzyme did not fluoresce when exposed to ultra violet light. However, after standing at room temperature for several days or addition of solid ammonium sulphate at pH 4·0, a fluorescence was apparent. These results would suggest the flavin prosthetic group of the enzyme is normally tightly bound, but under the conditions described above, is split off from the apo-enzyme and is free to fluoresce.

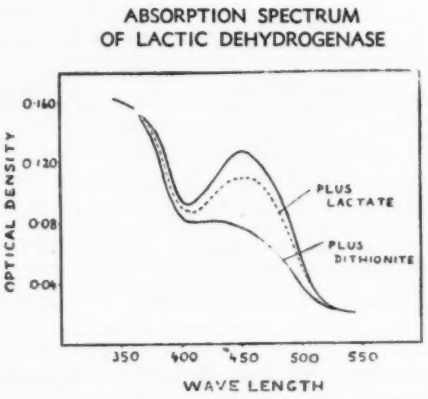


Fig. 4. *Absorption spectrum of the lactic dehydrogenase.* 1 ml. of the 20-fold purified enzyme (5 mg. protein/ml.) was placed in a 10 mm. Beckman quartz glass cuvette with a Perspex insert described by Martin and Morton (1956b). Solid L-lactate and sodium hydrosulphite were used for reduction.

DISCUSSION.

The role of flavins in the metabolism of Lactobacillus arabinosus.

The high amounts of flavin found in *Lactobacillus arabinosus* in the absence of detectable amounts of cytochromes, indicate an important role of the flavins in electron transport in this organism. A similar conclusion has been reached recently by Strittmatter (1958) in a survey on electron transport in the *Lactobacilli*.

The increased flavin content of *Lactobacillus arabinosus* when grown under anaerobic conditions compared with aerobic conditions (Table 1) is similar to the results found by other workers. Peel (1958) found that *Saccharomyces cerevisiae* grown under anaerobic conditions contained 35 p.c. more flavin than when grown under aerobic conditions. Pett (1935) and Singh, Agarwal and Peterson (1948) had found similar increases in the flavin content of *S. cerevisiae* grown anaerobically. Peel (1958) found that, in general, higher flavin contents were associated with anaerobic growth.

The increased specific activity of the flavokinase and adenyl-transferase of *Lactobacillus arabinosus* when the organism was grown under anaerobic conditions compared with aerobic growth (Table 2) agrees with the increased flavin content of the organism under these conditions.

In this paper evidence has been presented which indicates that the lactic dehydrogenase of *Lactobacillus arabinosus* is a flavoprotein. Dolin (1953)

showed that DPNH-menadione reductase of *Streptococcus faecalis* was a flavoprotein. The same worker (Dolin, 1955) also showed that the diaphorase of this organism was a flavoprotein enzyme. Thus it was quite possible that the DPNH-menadione reductase and diaphorase of *Lactobacillus arabinosus* were also flavoproteins. Lactic dehydrogenase, DPNH-menadione reductase and diaphorase were thus considered as representative of flavoprotein enzymes in *Lactobacillus arabinosus*.

The increased activity of lactic dehydrogenase and DPNH-menadione reductase compared with the more or less constant activity of the two non-flavoprotein enzymes pyruvic reductase and glyceraldehyde-3-phosphate dehydrogenase (Table 3) suggests that the increased flavin content under anaerobic conditions may be paralleled by a similar increase in flavoprotein enzymes. There is no apparent explanation for the decrease in diaphorase activity under anaerobic conditions although Lenhoff *et al.* (1956) found the diaphorase content of *Pseudomonas fluorescens* decreased when the organism was grown at low oxygen tensions.

The results just discussed serve to stress that flavoproteins may be of particular significance in electron transport in *Lactobacillus arabinosus* under anaerobic conditions. Also the presence of nitrate and fumarate reductase (both flavoprotein enzymes in anaerobic organisms, Nicholas and Nason, 1955; Warringa *et al.*, 1958) in cell-free extracts of *Lactobacillus arabinosus*, suggests these enzymes may act as terminal respiratory enzymes under anaerobic conditions. Although no direct coupling of the enzymes with the lactic dehydrogenase could be demonstrated in cell-free extracts. The role of flavoproteins in electron transport and the details of the electron transport chain in *Lactobacillus arabinosus* are at present under investigation.

The role of lactic dehydrogenase in the metabolism of Lactobacillus arabinosus.

Evidence presented in this paper indicates that this enzyme, in *Lactobacillus arabinosus*, is a flavoprotein which oxidizes L-lactate to pyruvate like the yeast lactic dehydrogenase (Appleby and Morton, 1954). The enzyme has an absorption spectrum resembling other flavoproteins, but the important feature of this spectrum was the reduction of the absorption peak at 450 mμ by the addition of lactate. This indicated that oxidation of the lactate resulted in reduction of the flavin prosthetic group of the enzyme. The increase in FMN/protein during the various stages of purification of the enzyme (Table 4) with a decrease in FAD/protein after the first stage, suggested the prosthetic group may have been FMN. Also, FMN was more effective than FAD in reversing the inhibition of enzyme activity caused by atabrine, again suggesting FMN may have been the prosthetic group. Final characterization of the prosthetic group must await the preparation of the enzyme in a pure

form. However, it is interesting to note that two other enzymes, prepared from micro-organisms, which oxidize lactic acid, contain FMN as a prosthetic group, e.g. the yeast lactic dehydrogenase of Appleby and Morton (1954; a flavohaemoprotein, cytochrome b_2) and the lactic oxidative decarboxylase of *Mycobacterium phlei* (Sutton, 1957).

The fact that the enzyme does not normally fluoresce when exposed to ultra violet light suggests that the prosthetic group may be linked to the protein through the N^3 position of the isoalloxazine ring as in the case of the old yellow enzyme (Theorell and Nygaard, 1955).

The demonstration of the flavoprotein lactic dehydrogenase in *Lactobacillus arabinosus* may well be of particular significance in considering the metabolism of the growing organism. Pyruvic acid produced by the glycolysis of glucose is converted to lactic acid in the presence of pyruvic reductase and reduced DPN. Lactic acid is the major end product of this organism, and in resting cells there is almost a quantitative conversion of glucose to lactate, but in growing cells there is only a 56 p.c. conversion of glucose to lactate under anaerobic conditions. Under growing conditions portion of the glucose would be utilized for the production of new cell material and pyruvate would be required as a source of acetyl-CoA for synthetic reactions, such as the synthesis of long chain

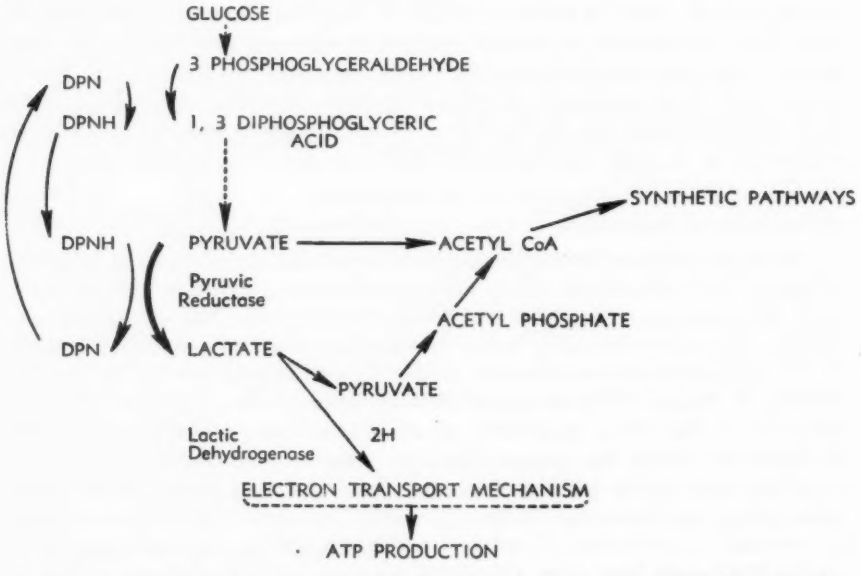


Fig. 5. A proposed scheme of metabolic pathways indicating the role of the lactic dehydrogenase in the metabolism of *Lactobacillus arabinosus*.

fatty acids and sterols. However, pyruvic reductase, which is the most active enzyme found in *Lactobacillus arabinosus*, would tend to convert any pyruvate formed to lactate. The flavoprotein lactic dehydrogenase could overcome this difficulty by regenerating pyruvate, which could be subsequently converted to acetyl-CoA and used for synthetic purposes. Hydrogen removed from the lactate could then pass into an electron transport chain at the flavin level (and thus not be available for the reduction of DPN) and possibly the energy so released could be used in the production of ATP. Thus lactate, which would be a wasteful end product, in energetic terms, might well provide a source of energy required for the growing cell. The mechanism outlined above is shown diagramatically in Fig. 5.

It may be noted that this mechanism will proceed under completely anaerobic conditions (using terminal respiratory enzymes as discussed above), because the pyruvic hydrogenase gives rise to oxidized DPN, required for further glycolysis, before the lactic dehydrogenase converts the lactate to pyruvate then available for acetyl-CoA production.

Acknowledgment. The author is indebted to Prof. R. K. Morton for helpful advice and discussion during the course of this work.

REFERENCES.

Appleby, C. A., and Morton, R. K. (1954): Nature, 173, p. 749.
Barker, S. B., and Summerson, W. H. (1941): J. biol. Chem., 138, p. 535.
Burch, H. B., Bessey, C. A., and Lowry, O. H. (1948): *Ibid.*, 175, p. 457.
Cousins, F. B. (1956): Biochem. J., 64, p. 297.
DeLuca, C., Weber, M. M., and Kaplan, N. O. (1956): J. biol. Chem., 223, p. 569.
Dolin, M. I. (1953): Biochim. Biophys. Acta, 15, p. 153.
Dolin, M. I. (1955): Arch. Biochem. and Biophys., 55, p. 415.
Edelhoch, H., Hayaishi, O., and Teply, L. J. (1952): J. biol. Chem., 197, p. 97.
Edson, L. (1954): Biochem. J., 41, p. 145.
Eichel, H. J. (1956): Biochim. Biophys. Acta, 22, p. 571.
Haas, E. (1944): J. biol. Chem., 155, p. 321.
Hager, L. P., Geller, D. M., and Lipmann, F. (1954): Fed. Proc., 13, p. 734.
Kornberg, A. (1955): Methods in Enzymology, S. P. Colowick and N. O. Kaplan, Edts. Academic Press Inc. Pub., New York, Vol. 1, p. 441.
Lenhoff, H. M., Nicholas, D. J. D., and Kaplan, N. O. (1956): J. biol. Chem., 220, p. 983.
Martin, E. M., and Morton, R. K. (1956a): Biochem. J., 62, p. 696.
Martin, E. M., and Morton, R. K. (1956b): *Ibid.*, 64, p. 221.
Nicholas, D. J. D., and Nason, A. (1955): J. Bact., 69, p. 580.
Nossal, P. M. (1953): Austral. J. exp. Biol., 31, p. 583.
Peel, J. L. (1958): Biochem. J., 69, p. 403.
Pett, L. B. (1935): *Ibid.*, 29, p. 937.
Robinson, R. W., and Hogden, C. G. (1940): J. biol. Chem., 135, p. 707.
Singh, K., Agarwal, P. N., and Peterson, W. H. (1948): Arch. Biochem. and Biophys., 18, p. 181.
Snoswell, A. M. (1957): Austral. J. exp. Biol., 35, p. 427.
Strittmatter, C. F. (1958): Fed. Proc., 17, p. 318.

Sutton, W. B. (1957): J. biol. Chem., 226, p. 395.
Theorell, H., and Nygaard, A. P. (1955): Acta Chem. Scand., 9, p. 1587.
Warringa, M. G. P. J., Smith, O. H., Giuditta, A., and Singer, T. P. (1958): J. biol. Chem., 230, p. 97.
Wosilait, W. D., and Nason, A. (1954): *Ibid.*, 208, p. 785.
Yamamura, Y., Kuranose, M., and Kuranose, E. (1952): J. Biochem., Japan, 39, p. 227.

AN ELECTRON MICROSCOPICAL STUDY OF GLOMERULAR CHANGES IN EXPERIMENTAL HYDRONEPHROSIS IN RATS

by R. K. F. PAK POY AND J. S. ROBERTSON[1]

(From the Department of Pathology, University of Adelaide).

(*Accepted for publication 2nd October, 1958.*)

SUMMARY.

An electron microscopical study of the glomerular changes in experimentally induced hydronephrosis in rats revealed a moderately complex mechanism of collapse of the capillaries. The hyalinised glomerulus appears to be formed by collapse of the capillaries with coalescence of thickened capillary basement membrane and loss of the cellular elements of the glomerulus. In the majority of glomeruli the peri-glomerular thickening is due to an increase in the thickness of the capsular basement membrane.

INTRODUCTION.

Numerous workers have shown that the kidney continues to secrete urine in spite of a complete blockage of the ureter, irrespective of whether the blockage is sudden or gradual (Hinman and Lee Brown, 1924; Johnson, 1932; Hinman, 1945; Persky *et al.*, 1955; Murnaghan, 1958). With the development of hydronephrosis, the renal parenchyma diminishes greatly in amount, due mainly to tubular atrophy and degeneration. Even at a very late stage the glomeruli in the light microscope appear little altered, with a relative increase in number, but eventually with collapse of the capillaries they become non-functioning hyaline masses (Allen, 1951; Bell, 1947) and there is marked replacement fibrosis of the nephrons. It was thought, therefore, that an electron microscopical study might shed further light on the mechanisms of collapse of the capillaries, and on the morphological changes associated with loss of function of the glomerulus.

MATERIALS AND METHODS.

The left ureters of rats were surgically occluded by a silver clip and the animals were killed at varying intervals. Portions of the kidneys were prepared for electron microscopy as in previous work (Pak Poy, 1958) using 1 p.c. osmium tetroxide and fixing for one hour at 4° C. Glomeruli were selected for thin sectioning by cutting off a thicker section (1 μ to 3 μ) from the block face and examining it by light microscopy. These sections were stained with hematoxylin and eosin, after removal of the methacrylate with toluene (10 seconds in Harris' Alum Hematoxylin and 5 seconds in 10 p.c. eosin). The thin sections were examined in a Philips electron microscope type EM100.

[1] Aided by a grant from the National Health and Medical Research Council.

RESULTS.

Macroscopically, the renal pelvis and calcyeal system reach maximum dilatation after twenty weeks, at which stage the renal parenchyma is a paper-thin shell. After this time, the size diminishes as the fluid is reabsorbed and presumably production diminishes. In these hydronephrotic kidneys three types of glomeruli are present—

Type 1. Its morphology suggests that the glomerulus is functioning.

Type 2. The glomerulus on morphological grounds appears to be functional, but there is a rupture in Bowman's capsule.

Type 3. Non-functioning glomerulus.

Type 1.

Glomeruli which appear to be functional from their morphological appearances, are present in all kidneys examined—the last animal studied was killed twenty-one weeks after the ureter was tied. In these glomeruli, a variable number of capillary loops show different degrees of collapse with corresponding loss of their lumens. In the early stages of collapse, the peripheral segment of the capillary loop loses its approximately circular outline and becomes U-shaped

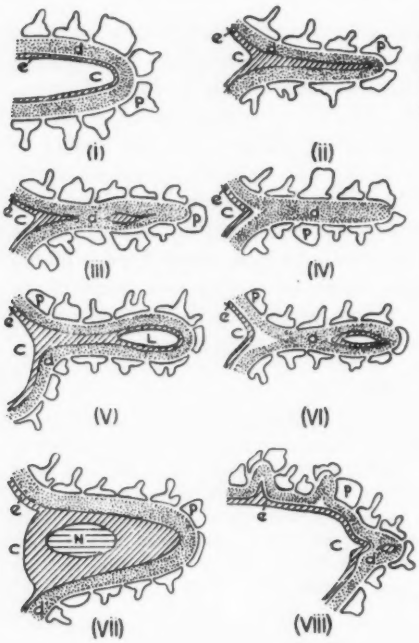


Fig. 1. In this Figure—c = patent capillary lumen, e = endothelium, d = dense layer of basement-membrane of capillary, p = pedicel, L = patent lumen in collapsed segment, N = nucleus of endothelial cell.

(i) This shows the early stage of collapse. The segment becomes U-shaped.

(ii) The segment has completely collapsed trapping a mass of endothelium which is continuous with the endothelial lining of the patent lumen.

(iii) A small mass of endothelium is trapped in a collapsed segment. Continuity with the endothelial lining of the patent lumen is lost.

(iv) There is a complete absence of endothelium in the collapsed segment with coalescence of the basement membrane.

(v) A small patent lumen (i) is still present in the collapsed segment. The endothelial layers lining the two lumens are continuous. This continuity is lost in (vi).

(vii) The capillary segment has collapsed down on an endothelial cell.

(viii) This shows the wavy appearance of the capillary wall when very small segments are involved.

(Fig. 1). The next stage is the approximation of the limbs of the U, with complete or partial loss of the lumen in that region (Figs. 2-4). Fig. 1 (ii) to (vii) illustrate six different arrangements this final stage may produce. If only small segments of the capillary wall are involved in the collapsing process, the basement membrane takes on a wavy, undulating appearance (Fig. 1 (viii)).

In many of these functioning glomeruli, Bowman's capsular basement membrane is replaced by a much thicker layer (as thick as 20,000A), composed of a mixture of dense homogeneous material and circularly arranged fibrils, with mottled material of lesser density filling the intervening spaces. These dense fibrils are similar to those seen in Bowman's capsular basement membrane of the normal mammalian glomerulus, in which it is clearly seen that the dense homogeneous basement membrane breaks up to form the fibrils (unpublished data). Banding could not be made out on these fibrils. Occasionally scattered amongst the dense material of the basement membrane are cells or projections of cytoplasm of uncertain nature. In other glomeruli, the capsular basement membrane is only slightly thickened and there are numerous collagen fibres in the adjacent surrounding interstitial space (Fig. 5). Some collagen fibres are present in the basement membrane of the capsule. Although generally flattened, the parietal epithelial layer occasionally gives rise to large projections, which rest on the basement membrane of abnormally close capillary loops (Fig. 7). In other instances when the capillary loop is close to the capsule, the visceral epithelium is forced against the parietal epithelium and the cell membranes appear to come into contact with one another. In light microscopy of adjacent thicker sections these areas appear as capsular adhesions. In one instance, one of these parietal projections gives rise to small cytoplasmic extensions, which rest in a pedicellar-like fashion on Bowman's capsular basement membrane a little distance away from the parent mass of parietal epithelium (Fig. 7). In one glomerulus we studied, around which the endothelium of a peri-glomerular capillary was in contact with the capsular basement membrane, the parietal epithelium was composed of cells lying free in Bowman's space, giving rise to trabeculae and pedicels which rest on the capsular basement membrane, as well as on the adjacent glomerular capillary loops (Fig. 8). The arrangement was similar to and as complex as in the visceral epithelial layer.

The surface of the parietal cell exposed to Bowman's space is generally smooth, but in a few areas there are small irregularities. In the cytoplasm there are pale areas of diminished density, comparable to that of Bowman's space, which are surrounded by dense thin membranes and may be larger than 1 μ in diameter. The walls of these sacs are irregular, with small projections into the lumen (Fig. 6), and because of the plane of sectioning some of these projections may appear as small masses of cytoplasm lying free in the sacs. Also in the cytoplasm adjacent to the basement membrane, there is an in-

Fig. 2. The micrograph shows the early stage of collapse of a segment of a capillary. The reduced lumen (c) is still completely lined by endothelium (es). Pedicels (p) are in Bowman's space. x 20,000.

Fig. 3. The segment of the capillary loop has completely collapsed with loss of the lumen. The arrow indicates a region where endothelium is absent, and the basement-membranes (d) of the limbs are in contact with one another. The endothelium (es) appears to have withdrawn from the collapsed segment and is lining the patent lumen (c) of the capillary. A small mass of endothelial cytoplasm (e) has been trapped in the terminal portion of the collapsed segment. Pedicels (p) are in Bowman's space. x 20,000.

Fig. 4. There is a completely collapsed segment of a capillary at (x) with coalescence of the basement-membrane. The endothelium is absent from the collapsed segment. The arrow indicates a region of collapse with loss of the endothelium, but the terminal portion of the segment (l) is still patent and the lumen is lined by endothelium (es). The cytoplasm of the endothelial cell (e) is present in a small collapsed segment. Patent capillary lumens are at (c) and pedicels are at (p). x 20,000.

Fig. 5. The cytoplasm (b) of a parietal epithelial cell contains masses of dense material (x) in the regions adjacent to the basement-membrane (d). The basement-membrane is irregular and slightly thickened with a mottled appearance. Collagen fibres (c) are present in the adjacent interstitial tissue and they can be seen invading the basement-membrane in the region to the right of (y). x 20,000.

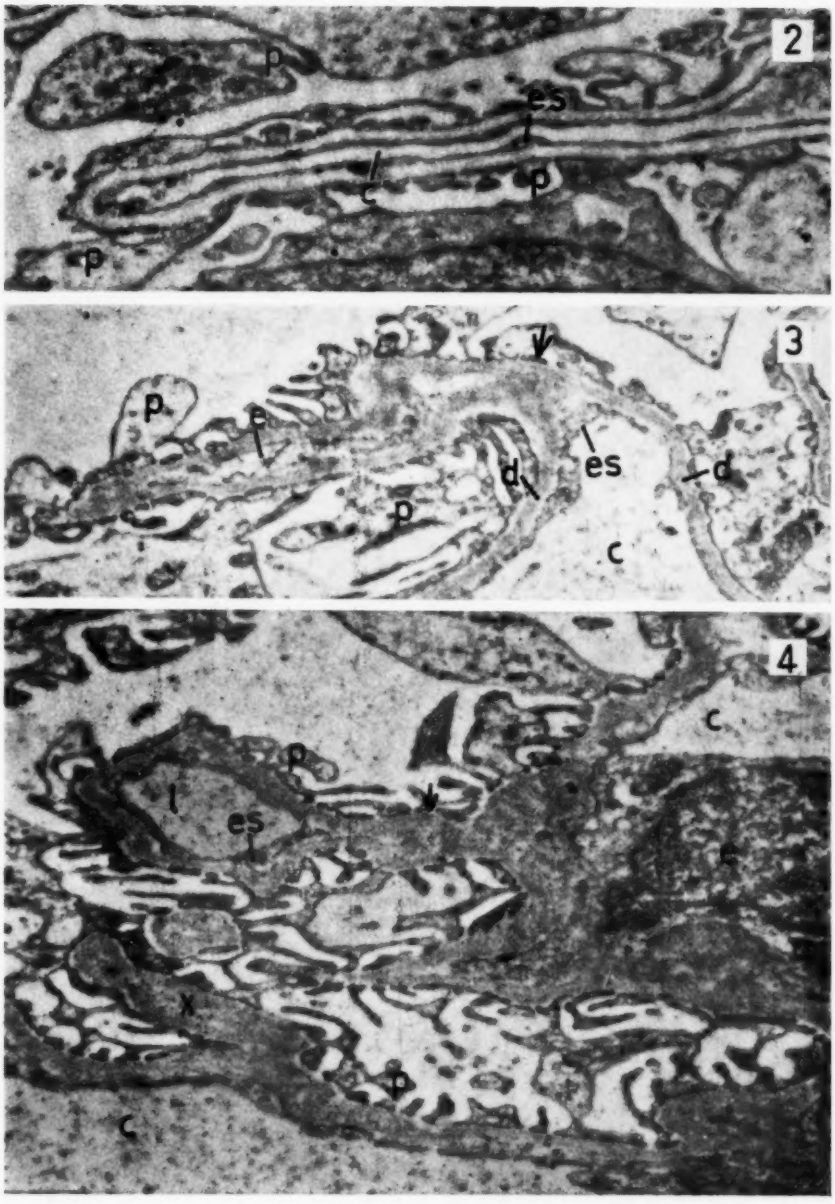
Fig. 6. In the parietal epithelial cytoplasm (b) there are clear spaces (o) lined by a membrane. The inner surface of the sacs is very irregular with many projections into the lumen, some of which appear to be lying free. This latter feature is probably due to the plane of sectioning. Bowman's space is at (s) and the basement-membrane (d) has a tendency to fibrillation. x 22,000.

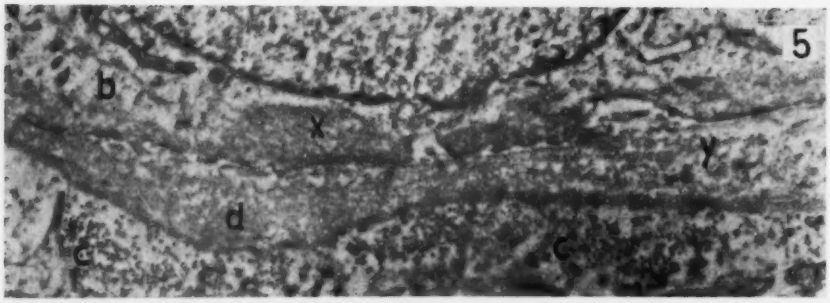
Fig. 7. The parietal epithelium lining Bowman's capsule gives rise to numerous projections (b) which rest on an adjacent capillary (c). In the lower right corner one of the masses of parietal epithelium gives rise to pedicellar-like structures (g) which rest on the capsular basement-membrane (d). The basement membrane has a fibrillary appearance. Bowman's space is at (s). x 17,000.

Fig. 8. Glomerular capillaries (c) in Bowman's space (s) are on the right half of the electron micrograph. Bowman's capsular basement-membrane (d) is just to the left of centre and at (k) there is a deep invagination of Bowman's capsule into Bowman's space. The parietal epithelium is composed of epithelial cells (z) lying free in Bowman's space, trabecula (v) and pedicels (b) which rest on the capsular basement-membrane (d). The periglomerular capillary (pg) containing red cells (r) is on the left and its attenuated endothelium (a) is separated from the capsular basement-membrane (d) by a thin, less dense, layer. In some areas the endothelium is in contact with the basement-membrane. The endothelium does not enter the invagination (k) of Bowman's capsule. Visceral epithelial cells are at (e) and interstitial stroma of the kidney at (i).

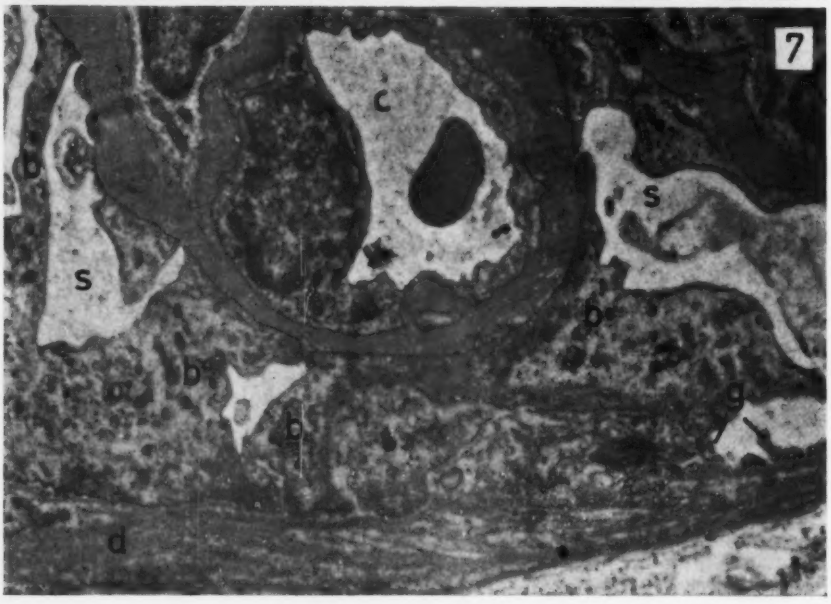
Fig. 9. This low power electron micrograph shows Bowman's capsular basement-membrane (d) in the top right-hand corner and fragments of the capsular epithelium (b) in the lower left-hand corner, with a rupture in the capsule between these two points. At the site of rupture there are degenerating cytoplasm (w) and vessels (v). The glomerular capillaries are at (c) and most of the visceral epithelium is lost from the outer surface of the capillary adjacent to the rupture. Portion of a tubule (t) is at the bottom right-hand corner and a mass of dense basement-membrane material is at (x).

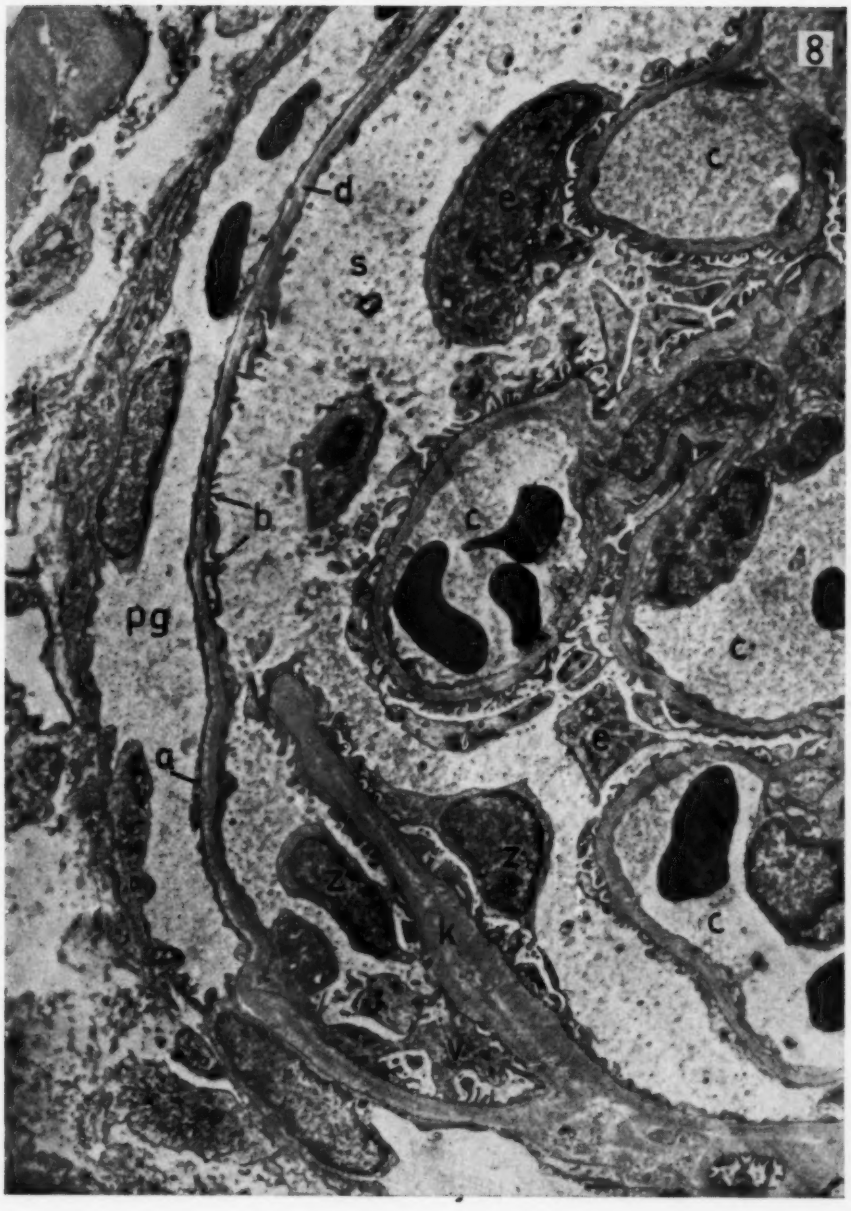
Fig. 10. This is a low power electron micrograph of a hyalinised glomerulus, composed of dense basement-membrane-like material (d), formed by thickening and coalescence of the basement-membrane of collapsed capillaries. Most of the cellular elements have disappeared although a few visceral epithelial cells (p) are still present. The nature of cell (m) in the middle of the mass is unrecognisable. In Bowman's space (s) there are small collections of collagen fibres (x) at the periphery of the mass of basement-membrane-like material.


2
p
es
l
c
p
p
3
p
e
l
d
es
d
p
c
4
c
p
l
es
x
p
c

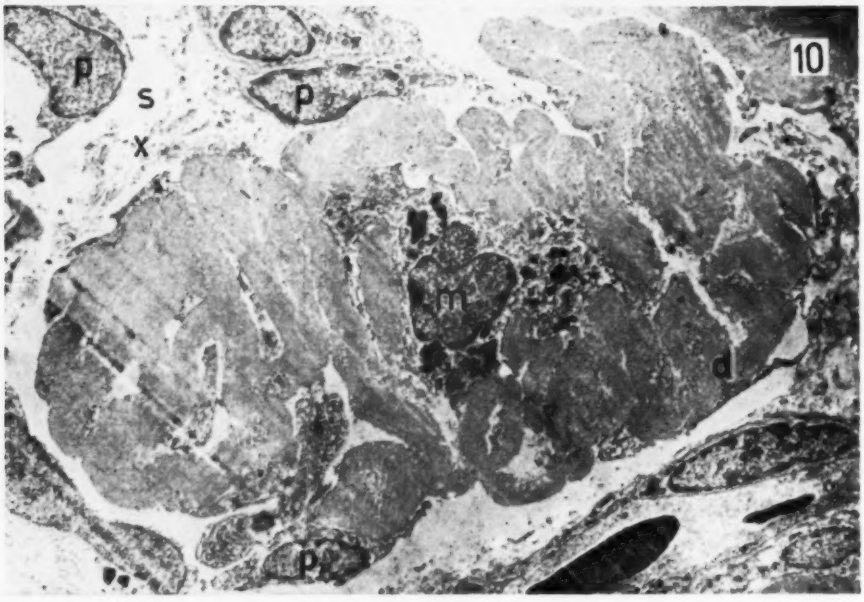

5
b
x
y
d
c
c

6
s
o
b
o
o
d


7
c
b
s
s
b
b
g
b
d


8
d
s
c
e
b
pg
a
z
k
v

9
c
a
d
v
w
b
x
v


10
p
s
x
p
m
d
p

creased amount of dense homogeneous material similar to that found in the normal glomeruli (Fig. 5).

At the hilar region, if Bowman's capsule is composed of fibrils, the dense homogeneous component adjacent to the adventitial coat of the arterioles appears to form the major component of the capillary loop basement membrane. However, when the basement membrane of the capsule is composed of dense homogeneous material, this membrane seems to form the major portion of the capillary loop basement membrane.

Furthermore, the visceral epithelial cells seem to form a more complex pattern than is present in the normal mammalian glomerulus (Hall, 1953; Pease, 1955; Yamada, 1955). In general, the endothelium appears normal and the amount of intercapillary tissue present is slightly increased. Due to the collapse of capillaries many more cells appear to be intercapillary in position (Pak Poy, 1958), but on careful examination some part of the cell can be found exposed to blood, thus indicating its endothelial nature. The inner less dense layer of the capillary wall (Pease, 1955; Yamada, 1955) is more frequently absent than present.

Type 2.

In the kidneys of animals killed about the 13th week after ligation of the ureter, the capsules of some of the glomeruli are ruptured, exposing the capsular space to the interstitial stroma of the kidney. The gap is usually small, but it may extend over a quarter of the capsule of the plane of sectioning, and in the gap are fibrils, blood vessels and pale swollen cytoplasm, containing a sparse number of organelles. The fibrils are banded and have the morphological appearance of collagen fibres but, unfortunately, it was not possible to make accurate measurements of the periodicity. At the margins of the gaps the parietal epithelium is usually absent over a large area, and if present it is usually much fragmented, although the capsular basement membrane is frequently recognisable with its free end projecting into the interstitial stroma.

The glomerular capillaries in this second type of lesion are moderately collapsed, but their lumens still contain blood cells. In the vicinity of the capsular defect, the visceral epithelial pedicels covering the outer surface of the capillaries may be absent (Fig. 9) and some of the adjacent pedicels tend to have larger bases resting on the capillary walls. Occasionally an epithelial cell undergoing degeneration is present. Away from the defect the visceral epithelium appears relatively normal.

Type 3.

Light microscopic examinations of kidneys in which hydronephrosis had been present for more than twenty weeks, revealed the presence of hyalinised glomeruli. In electron micrographs, the hyaline ball appears as a homogeneous mass of greatly thickened compressed basement membrane from a capillary loop

with coalescence in many places. Scattered in and around this mass, there may be a few cells, which can occasionally be recognised as endothelial or epithelial in nature (Fig. 10). Remnants of Bowman's capsule may still be present, and this helps considerably in the recognition of these structures as being of glomerular origin. There are a number of banded collagen fibres in the capsular defect and around the periphery of the dense mass.

DISCUSSION.

Hyalinisation of the glomerulus is a non-specific change described in numerous diseases of the kidney, but the detailed morphology of this process has not been elucidated. In electron microscopical studies of diseased glomeruli, Farquhar *et al.* (1957) described the hyalinised glomerulus of chronic glomerulonephritis as a mass of basement-membrane-like material. In this study, it appears that hyalinisation is a degenerative process associated with loss of function of the glomerulus. At first there is collapse of the capillary loops with some coalescence of the basement membrane. This step is followed by rupture of Bowman's capsule, through which grows connective tissue from the surrounding interstitial stroma. Further collapse of the capillaries follows, with great diminution of the cellular elements, and further coalescence and some increase of basement membrane material. At this stage Bowman's capsule is only just recognisable, but is helpful in the identification of the structure. Collagen fibres are now much closer to the mass of basement-membrane material, but in the most advanced lesion examined they were not present in the mass. We suggest that eventually connective tissue overgrows the mass so that it can no longer be differentiated from the interstitial stroma, in which there are present irregular small masses of dense basement-membrane-like material.

Electron micrographs of hyalinised glomeruli obtained from an autopsy on a patient suffering from long-standing hypertension, and from a biopsy taken at operation from a kidney containing calculi with superadded hydronephrosis, pyelonephritis and peri-nephritis, revealed collagen fibres scattered in the mass of basement-membrane-like material (unpublished data).

In the majority of glomeruli the thickening of Bowman's capsule appears to be due to increase of basement-membrane material, which is a specialised form of intercellular material. It is possible that these fibres of dense material are the electron microscopical counterparts of the argyrophilic fibres described in light microscopical studies by McGregor (1929) and by Moritz and Hayman (1934). In their study on the disappearance of glomeruli, Moritz and Hayman described an ingrowth of the argyrophilic component of the capsular basement-membrane into the hyalinised mass. We did not see evidence to suggest that any component of the capsular basement membrane is involved in the above process.

Not much functional importance has been attached to the parietal epithelium lining Bowman's capsule although it has the same origin as the visceral epithelium (Herring, 1900). It is not surprising therefore that in capsular adhesions, the parietal cells give rise to pedicels which rest on the capillary walls. The pedicellar arrangement of the parietal epithelium in the glomerulus surrounded by a peri-glomerular capillary affords perhaps some indirect evidence in support of the hypothesis that the pedicellar system is important in fluid and metabolite transfer across the capillary wall.

It has been suggested that one mechanism by which the urine is reabsorbed in hydronephrotic kidneys is the formation of high-and-low pressure glomeruli. The urine is secreted in the high pressure glomeruli and then escapes into the interstitial spaces because the tubules have degenerated. From the interstitial space the urine finds its way to the low pressure glomeruli where it is reabsorbed back into the bloodstream (Hinman, 1945). The presence of ruptured capsules in a number of glomeruli in which the capillaries appear to be still functional might conceivably support such a hypothesis.

Acknowledgments. We should like to thank Dr. S. G. Tomlin, Department of Physics, University of Adelaide, for granting access to the electron microscope, and Mr. A. Britten-Jones, Honorary Surgeon, Royal Adelaide Hospital, who provided the renal biopsy. The late Miss G. Walsh drew Fig. 1.

REFERENCES.

Allen, A. C. (1951): The Kidney—Medical and Surgical Diseases, Grune and Stratton, Inc., New York.
Bell, E. T. (1947): Renal Diseases, Lea and Febiger, Philadelphia.
Farquhar, M. G., Vernier, R. L., and Good, R. A. (1957): J. exp. Med., 106, p. 649.
Hall, B. V. (1953): Proc. 5th Ann. Conf. Nephrotic Syndrome, Josiah Macy, Jr., Foundation, Philadelphia.
Herring, P. T. (1900): J. Path. Bact., 6, p. 457.
Hinman, F. (1945): Surgery, 17, p. 836.
Hinman, F., and Lee Brown, R. K. (1924): J. Amer. med. Ass., 82, p. 607.
Johnson, C. M. (1932): J. Urol., 27, p. 279.
McGregor, L. (1929): Amer. J. Path., 5, p. 545.
Moritz, A. R., and Hayman, J. M., Jr. (1934): *Ibid.*, 10, p. 505.
Murnaghan, G. F. (1958): Postgrad. med. J., 34, p. 143.
Pak Poy, R. K. F. (1958): Amer. J. Path., 34, p. 885.
Pease, D. C. (1955): J. Histochem. Cytochem., 3, p. 295.
Persky, L., Storasli, J. P., and Austen, G., Jr. (1955): J. Urol., 73, p. 740.
Yamada, E. (1955): J. biophysic. biochem. Cytol., 1, p. 551.

THE EFFECT OF SALICYLATE ON THE *IN VITRO* ANTIGEN-ANTIBODY REACTION IN ANAPHYLAXIS[1]

by E. R. TRETHEWIE AND C. RUMBERG

(From the Physiology Department, University of Melbourne).

(*Accepted for publication 20th October, 1958.*)

SUMMARY.

A light scattering photometer was used to measure the change in turbidity on—

(i) adding antigen to sensitized guinea-pig serum and inhibiting this reaction with sodium salicylate;
(ii) diluting serum with distilled water; and on,
(iii) adding sodium salicylate to serum.

This work indicates that the antibody antigen reaction with rise in turbidity and the sodium salicylate "salting in" reaction (including $A_B : A_G$ inhibition) with fall in turbidity complement each other with the final turbidity value depending on the concentrations of sodium salicylate, egg albumin and serum. It indicates salicylate is effective in *in vitro* systems.

INTRODUCTION.

Sodium salicylate has been found to inhibit the precipitation of sensitized rabbit serum by the addition of antigen – either egg albumin or horse euglobulin – as determined by a micro-chemical method (Coburn and Kapp, 1943). Preliminary investigations (Trethewie, 1954a) showed that salicylate in more physiological dosage, inhibited the polymerisation or union of antigen-antibody as determined by turbidity studies. These studies were carried out using guinea-pig serum as antibody and egg albumin as antigen, estimations being made on a photometer. These studies are extended in the present paper.

The particular dose of salicylate chosen was close to that level used to inhibit histamine release from the anaphylactic guinea-pig lung (Trethewie, 1951).

Thus the rationale of the experiment was that if the anaphylactic reaction is due to antibody antigen interaction, and its inhibition by salicylate due to an influence on this interaction, *in vitro* inhibition might be demonstrable with physiological dosage.

---

[1] Technical assistance for this work was aided by a grant from the National Health and Medical Research Council.

METHODS.

Sensitive serum. Guinea-pigs, in batches of 14-21, were injected subcutaneously each with 15 mg. of egg albumin dissolved in 0·5 ml. of sterile saline. All injections were given on the right anterior abdominal wall after an area of 4 cm. square had been clipped of hair. On sensitization the guinea-pigs weighed between 100-170 gm. They were then isolated and sexed when necessary for periods varying from 10-44 days, after which time they were bled.

The weights of the non-sensitized pigs used were comparable to the weights of the sensitized pigs on the day of bleeding. The pigs were bled following a stunning blow on the back of the head. The fur was clipped from the ventral wall of the neck, the jugular veins exposed and severed with a scalpel. The pigs were held legs uppermost so that the animal bled freely into a test tube for a minute. There was one test tube per animal. The tubes were then plugged with cotton wool, incubated at 37° C. for one hour and placed in the refrigerator overnight. The following morning the serum was poured off, pooled and spun for 20 minutes at 2,500 r.p.m. to remove any blood cells.

The same morning, freshly prepared solutions of egg albumin and sodium salicylate in distilled water, serum, distilled water and normal saline were spun for one hour at 12,000 r.p.m. to remove all dust.

Details of Collection of Serum.

Exp. No.	No. pigs used	Weight on injection (gm.)	Weight on bleeding (gm.)	No. days between injection and bleeding	Yield of serum (ml.)
1	14	100-170	—	13(11)*10(3)	20
2	30	—	210-310	—	40
3	14	100-160	320-390	44	30
4	17	100-150	160-250	14	35
5	19	110-155	150-220	10	33
6	14	—	250-300	—	33

* Numbers in brackets refer to the number of pigs.

Turbidity measurements. Turbidity was measured by using a Brice Phoenix Universal light scattering photometer as a turbidometer.[2] The instrument was used to measure intensities of direct light passing through solutions of serum $I_0°$, and intensities of light scattered at 90° to the light source $I_{90}°$.

Turbidity then equals $I_{90}°/I_0°$ which is proportional to solute particle size. The wavelength of the light source was 546A° (green). Light intensities were read as scale deflections on a galvanometer and the sensitivity was always adjusted so that at least one intensity covered more than 80 p.c. of the scale.

For exp. 1-4 and 6, a glass cell 24 × 24 mm. of fabricated optical plate with sinter fused joints was used and for exp. 5 the cell size was 33 × 33 mm. Care was taken to remove all dust from apparatus by washing the cell and pipettes several times in dust-free distilled water.

Serum (5-7·5 ml.) was added to the cell first followed by either saline or distilled water. Additions of egg albumin or sodium salicylate or distilled water were kept to values of 0·5 ml. or 0·2 ml. so that the dilution effect on turbidity would be slight. In between each

[2] Use of photometer kindly allowed by C.S.I.R.O. Wool Textiles.

experiment the cell was washed with chromic acid for six minutes to ensure denaturation of proteins. Care was then taken to remove all chromic acid from the cell.

A corner of the cell was marked and this corner was placed in the same position in the photometer for each experiment.

Solutions. Sodium salicylate used was slightly impure as the pH of a 300 mg. p.c. solution was in the vicinity of 5·5. Crystalline egg albumin was prepared by Dr. Trautner of the Physiology Department. Solutions of egg albumin and sodium salicylate were prepared by dissolving the substances in distilled water.

Six experiments employing fourteen turbidity systems were set up using pooled serum. A stands for egg albumin; S stands for sodium salicylate; W stands for distilled water. pH was obtained using solutions from the cell. In experiments 1, 2, 4 and 6, the readings were 8·4, 8·3, 8·5 and 8·0 respectively for each system used. Control experiments: 2 and 6. Experiments with sensitized pigs: 1, 3, 4 and 5.

Sensitivity was checked in each batch of pigs by giving an intravenous jugular injection of egg albumin. Violent convulsions and death within a few minutes were found in all pigs of experiments 1, 3, 4 and 5 that were selected for test.

Turbidity measurements were read every 1-15 minutes; a graph was drawn. For convenience of tabulation values of turbidity were estimated from the graph.

Each addition of sodium salicylate increased the concentration of that salt by 100 mg. p.c.

The effect of adding 0·50 ml. of distilled water to serum was found to alter the turbidity by 0·01 units.

EXPERIMENTAL.

The effect of crystalline egg albumin on sensitized plasma.

Normal plasma shows little change in turbidity when crystalline egg albumin is added. This is shown in Table 1 and Table 2, where the change in turbidity was 0·07 in 50′, −0·02 in 21′, and −0·02 units in 17′, in three separate experiments. When albumin is added to sensitized plasma the turbidity rises, e.g. in exp. 1 the turbidity rose 0·51 units in 65′, in exp. 5, 0·17 in 23′ (Table 1), in exp. 3a it rose 0·27 units in 90′, and in exp. 4a it rose 0·50 units in 118′ (Table 2). The average rise with sensitized serum was 0·36 units and with control serum 0·01 units.

TABLE 1.

Change in Turbidity—Separate Systems.

Exp. No.	Time in minutes	Plasma + albumin	Plasma + salicylate + albumin	Degree of salicylate inhibition
(control) 2	50	0·07	−0·02	0·09
	50	nil added	nil added	0·04
1	65	0·51	0·19	0·32
5	23	0·17	—	—

+ = rise
− = fall (in turbidity)

TABLE 2.

Change in Turbidity—Salicylate Superadded

Exp. No.	Albumin	Fall with salicylate
(control) 6a	−0·02 (21′)	0·23 (26′)
(control) 6b	−0·02 (17′)	0·02 (8′)
3a	0·27 (90′)	0·19 (23′)
4a	0·50 (118′)	0·38 (45′)

When sodium salicylate is added in a concentration of 100 mg./100 ml. system (the pH remaining constant) to control non-sensitive serum to which albumin has been added at the same time there is a small fall in turbidity, e.g. in exp. 2, Table 1, the turbidity was 0·02 units less, 50′ after adding the albumin. Comparing this with the slight rise of 0·07 units for the serum to which albumin alone had been added, there was a net fall of 0·09 units in turbidity. When sodium salicylate is added in this concentration to sensitized serum to which albumin has been added at the same time the rise in turbidity due to albumin is largely inhibited. This is shown in exp. 1, Table 1, where the rise in turbidity for albumin added alone was 0·51 units, while in the presence of salicylate the rise was only 0·19 units, i.e. the inhibition due to salicylate was 0·32 units.

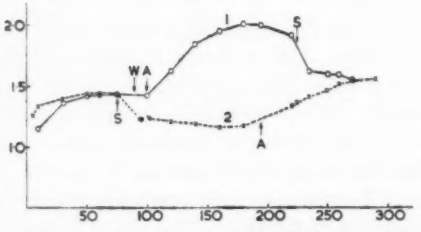


Fig. 1. Change in turbidity following addition of albumin (A) first and then salicylate (S) to sensitive serum, continuous line (———), compared with change in turbidity following addition of salicylate (S) first and then albumin (A) to sensitive serum, interrupted line (x - - - - - x). Ordinate: turbidity reading. Abscissa: time in minutes. At W 0·5 ml. distilled water added. pH of each system 8·5.

If salicylate is added some time after the albumin, to allow agglomeration of molecules, it is found that the albumin polymerisation is partly reversible. This is shown in Fig. 1 and Table 2. In Fig. 1 the final turbidity was unchanged whether albumin was added before or after the salicylate. In one instance the turbidity rose following albumin and then fell with salicylate (1, Fig. 1), and in the other instance there was a slight fall following addition of salicylate, followed by a slight rise after adding albumin (2, Fig. 1). In two control experiments (6a, 6b) there was a slight fall in turbidity following the

addition of albumin, 0·02 units in each instance. This occurred during 21 and 17 minutes respectively. After the addition of sodium salicylate the turbidity fell 0·23 units in 26′ in the one instance and only 0·02 units in the other instance. When albumin was added to sensitized serum, on the other hand (exp. 3a, 4a), the turbidity rose 0·27 units in 90′ and 0·50 units in 118′ respectively (Table 2). Following the addition of sodium salicylate there was a fall in turbidity in each instance of 0·19 and 0·38 units respectively.

DISCUSSION.

Both serum antibody and antigen appear to be multivalent with respect to each other (Gitlen and Edelhoch, 1951; Heidelberger and Kendall, 1935) and their interaction appears as a series of competing bimolecular reactions. Thus it would be expected that sodium salicylate would block both the reaction $A_B + A_G \rightarrow A_B \cdot A_G$ and the subsequent polymerisations.

Sodium salicylate inhibits the release of histamine from the anaphylactic guinea-pig lung (Trethewie, 1951) so that the inhibition of antibody antigen reaction by sodium salicylate is consistent with the theory that anaphylaxis is due to antibody antigen interaction.

The fact that *in vitro* inhibition of "polymerisation" or "lattice formation" of union of antigen antibody occurs, as indicated by the turbidity studies, suggests that salicylate has peripheral action in inhibiting antigen reaction. This is in agreement with the findings in the isolated perfused lung (Trethewie, 1951) and its significance as a peripheral inhibitor has been further outlined (Trethewie, 1954b, 1958; Hugues, Lecomte and van Cauwenberge, 1954). There is no evidence that the reaction occurs through ACTH release as was suggested originally by Hungarian workers.

REFERENCES.

Coburn, A. F., and Kapp, E. M. (1943): J. exp. Med., 77, p. 173.
Gitlen, D., and Edelhoch, H. (1951): J. Immunol., 66, p. 67.
Heidelberger, M., and Kendall, F. E. (1935): J. exp. Med., 62, p. 697.
Hugues, J., Lecomte, J., and van Cauwenberge, H. (1954): Arch. int. pharmacodyn., 100, p. 105.
Trethewie, E. R. (1951): Austral. J. exp. Biol., 29, p. 443.
Trethewie, E. R. (1954a): Lecture delivered at the University of Minnesota.
Trethewie, E. R. (1954b): Med. J. Austral., 1, p. 388.
Trethewie, E. R. (1958): Austral. J. exp. Biol., 36, p. 275.

THE EFFECTS OF HEAVY METAL IONS ON THE HUMAN ERYTHROCYTE

III. INHIBITION OF THE EFFECTS OF LEAD AND MERCURY

by P. C. VINCENT

(From the Department of Medicine in the University of Sydney and the Clinical Research Unit of the Royal Prince Alfred Hospital, Camperdown[1]).

(*Accepted for publication 27th June, 1958.*)

SUMMARY.

Thiosorbitol stoichiometrically inhibits the effects of mercury on the red cell, while cysteine is less effective in this regard.

Disodiumethylenediaminetetraacetic acid (SED) partly prevents the effects of subsequently added mercury, but fails to remove the metal from the cell, or reduce the loss of potassium, once combination is established.

SED completely prevents the effects of equimolar lead, and also removes most of the metal from the cells after combination is established.

In the latter instance, the lead-induced potassium efflux is only partly curtailed.

Pre-incubation of SED with red cells decreases its inhibitory capacity with respect to both lead and mercury. A similar effect following pre-incubation with calcium is described.

Fresh stromata, but not lipid-free stromatin or haemoglobin, compete equally with the red cell for the uptake of lead.

The implications of these findings are discussed.

INTRODUCTION.

Previous studies (Vincent and Blackburn, Vincent, 1958) have shown that the ions of several heavy metals interfere with the K^+ metabolism, shape and volume of the human red cell. A quantitative relationship between the K^+ efflux and both the concentration of metal and the duration of its action, was described. The present studies attempt to define the modes and sites of action of Pb and Hg by using substances expected to interfere with their actions.

It has been shown that both Pb and Hg become associated with the red cell, so that approximately 90 p.c. of either metal disappears rapidly from the

[1] Supported in part by a grant from the National Health and Medical Research Council.

suspending medium after mixing. This association persists at least until the efflux of K^+ has reached equilibrium and, in fact, it was not observed to dissociate in one hour's incubation at 37° C. The following questions, therefore, arise:

(1) Is the metal associated with the surface of the cell, or the cell membrane itself, or the interior of the cell, or is it distributed in all?

(2) Does the metal enter into chemical combination with components of the cell, or is it retained by physical association alone?

(3) Can the metal be recovered from the cell, and, if so, is the efflux of K affected by this removal?

Different opinions as to the site of binding of heavy metals will be found in the literature. Aub *et al.* (1925) suggested that Pb is bound to the red cell, after combination with inorganic phosphate, as a precipitate of the insoluble lead salt spread through the membrane. Jandl and Simmons (1957), on the other hand, believe that divalent metal cations associate with the red cell through carboxyl groups.

Two types of inhibitor have been studied; the first of these, represented by disodiumethylenediaminetetraacetic acid (SED), thiosorbitol and cysteine, are chelating agents which are known to combine with heavy metal ions in solution. Secondly, simple fractions of the red cell, such as stroma, lipid-free stroma and haemoglobin, were studied in competition with whole red cells for the uptake of Pb.

Methods.

The collection and preparation of normal human red cells, their incubation with heavy metals, and the assay of supernatant K^+ and Pb^{++} or Hg^{++}, were carried out as described previously. SED, thiosorbitol and cysteine were obtained pure and crystalline. Stromata were prepared by the methods of Ballantine (1944) and of Prankerd and Altman (1954) and rendered lipid-free by Ballantine's method. Drabkin's method was followed in the preparation of haemoglobin (Drabkin, 1949). Each preparation was thoroughly washed with isotonic NaCl and found to be free of K.

The substances being tested for their inhibitory effect were introduced to the red cell heavy metal reacting system either before, simultaneously with, or after the mixing of the cells with the metal. All appropriate combinations of cells, metal solutions, inhibitors and suspending fluid were set up as controls. Particular attention was paid to the effects of the inhibitors themselves on the red cells, especially with respect to SED, but in no case did they lead to a K^+ efflux.

EXPERIMENTAL.

Chelating Agents — Thiosorbitol, Cysteine and SED.

(a) *Thiosorbitol and Cysteine.* Thiosorbitol was added to suspensions of human red cells in varying quantities and the effects of Cd, Hg and Pb, added 30 minutes later, were compared with their effects in the absence of inhibitor.

TABLE 1.

Comparison of the inhibition of the effect of Cd, Hg and Pb on the red cell by thiosorbitol. Thiosorbitol added to cells 30 minutes before metal. Incubated at 37° C.

Metal	Conc. of metal μ moles/ml red cells (m)	Conc. of thiosorbitol (Ts) μ moles/ml red cells	Ts/M Ratio	K lost p.c. available		K_{ts}-K_m p.c. available
				K_{ts}	K_m†	
Cd	100	800	8	− 0·7		−10·0
		400	4	− 0·2		−9·5
		200	2	− 0·5		−9·8
		100	1	3·9		−5·4
		50	0·5	5·8		−3·5
		0	0		9·3	
Hg	0·2	1·6	8	0·2		−8·2
		0·8	4	0·8		−7·6
		0·4	2	0·1		−8·3
		0·2	1	1·5		−6·9
		0·1	0·5	3·8		−3·6
		0	0		8·4	0
Pb	0·1	0·8	8	17·3		−1·1
		0·4	4	13·2		−5·2
		0·2	2	16·7		−1·7
		0·1	1	11·0		−7·4
		0·05	0·5	15·1		−3·3
		0	0		18·4	0

† K_{ts}-K_m=difference between the K released after incubation with thiosorbitol and K released in the absence of thiosorbitol.

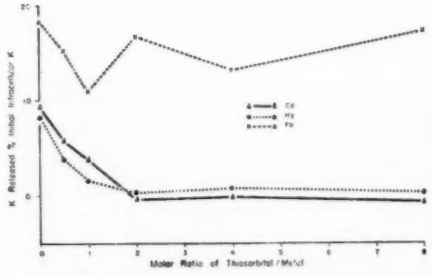


Fig. 1. *The inhibition by thiosorbitol of the effects of Cd and Hg on the red cell, and the lack of inhibition of Pb.* Thiosorbitol and cells were mixed and 30 minutes later Cd (100 µmoles/ml. packed cells), Hg (0·2 µmoles/ml. packed cells), or Pb (0·1 µmoles/ml. packed cells), was added and the whole incubated for 30 minutes at 37° C.

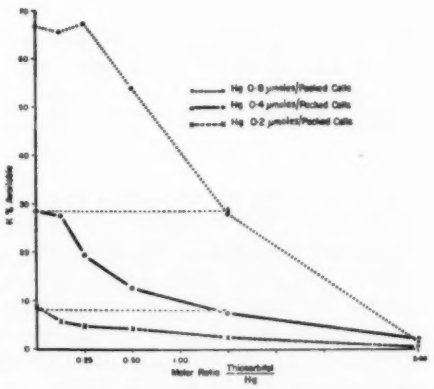


Fig. 2. *The release of K from the red cell after the addition of three different concentrations of Hg, partly inhibited by ratios of thiosorbitol to Hg of less than* 2:1. The horizontal dotted lines indicate the proportional inhibition by thiosorbitol in equimolar ratio (see text).

Table 1 and Fig. 1 show the K^+ lost from the cell, after the action of the 3 metals, in this type of experiment. These results indicate that the expected loss of K^+ from cells treated with 100 μmoles Cd or 0·2 μmoles Hg per ml. packed cells is completely prevented if not less than 2 moles of thiosorbitol per mole of metal are first mixed with the cells. Furthermore, molar ratios of thiosorbitol to metal of less than 2:1 partially prevent the loss of K from the cells. On the other hand, no concentration of thiosorbitol tested was effective in preventing the effects of Pb on the red cell.

The loss of K^+ in the presence of lower ratios of thiosorbitol/Hg than 2:1 is directly related to the Hg *not* bound by the inhibitor. Solutions of thiosorbitol equimolar with Hg would be expected to bind half-molar quantities of the metal. Table 2 shows that Hg mixed in these proportions does, in fact, lead to the same

TABLE 2.

Comparison of inhibition by thiosorbitol of the effect of 3 concentrations of Hg. Thiosorbitol added to cells 30 minutes before Hg. Incubated 30 minutes at 37° C.

Molar Ratio $\frac{\text{Thiosorbitol}}{\text{Hg}}$	K released, p.c. available		
	Concentration of Hg μm/ml packed cells		
	0·8	0·4	0·2
2·0 or 2:1	1·5	1·8	0·8
1·0 or 1:1	28·0	7·4	2·3
0·5 or 1:2	54	12·5	4·3
0·25 or 1:4	67·1	19·6	4·9
0·125 or 1:8	65·8	27·7	5·4
0 or 0	66·8	28·3	8·5

loss of K^+ from the red cell as that produced by half the concentration, without thiosorbitol. For example, 0·8 μmoles Hg/ml. packed cells in the presence of equimolar thiosorbitol, causes the loss of 28 p.c. of the available K^+, compared with a loss of 28·3 p.c. following exposure to 0·4 μmoles Hg. Similarly, 0·4 μmoles Hg with equimolar thiosorbitol causes a loss equivalent to 0·2 μmoles Hg without thiosorbitol (7·4 and 8·5 p.c. available K^+ respectively). In Fig. 2, the horizontal lines between the curves for different concentrations of Hg indicate this relationship.

Fig. 2 also shows that, as the concentration of Hg is increased, higher thiosorbitol/Hg ratios are required to produce a minimal effect. Thus, although a ratio of 1:8 significantly reduces the K^+ loss from cells treated with 0·2

μmoles Hg/ml. packed cells, such a reduction is only attained with ratios of 1:4 for 0·4 μmoles Hg and 1:2 for 0·8 μmoles Hg.

The effect of the time of adding thiosorbitol to the metal/red cell system was studied by adding excess thiosorbitol (thiosorbitol:Hg = 10:1) to the cells 60 seconds before, and 5 and 30 minutes after the addition of Hg. Addition of thiosorbitol immediately before the addition of metal was completely inhibitory to the release of K^+, but introduction of the inhibitor after the metal was without demonstrable effect.

Cysteine was studied in a similar manner and found to be ineffective against Pb, but effective against the action of Hg. It completely prevented the loss of K^+ if added to the red cells, in molar ratios of cysteine: Hg of 10:1, before the addition of Hg. It was ineffective if added to the cells after they had been exposed to Hg.

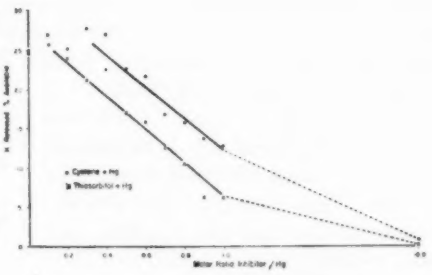


Fig. 3. *Comparison of the effectiveness of cysteine and thiosorbitol as inhibitors of the Hg effect.* Red cells, mixed with cysteine or thiosorbitol in the ratios shown, were incubated with 0·4 μmoles Hg/ml. packed cells for 30 minutes at 37° C.

Fig. 3 shows that cysteine is less effective than thiosorbitol in preventing the effects of 0·4 μmoles Hg per ml. packed cells. The only K^+ loss found in the presence of thiosorbitol is that due to the Hg which cannot be bound by that concentration of inhibitor; but in the case of cysteine, the K^+ loss is greater than this, although still related to the inhibitor concentration. This suggests that under these conditions the red cell can remove a fraction of the Hg from combination with cysteine, but not from combination with thiosorbitol. This finding is of value in assessment of the degree of affinity between Hg and the red cell surface, as will be discussed below.

(b) *SED.* It was necessary at the outset to determine whether a powerful chelating agent such as SED would itself exert any effect on the K^+ exchanges of the red cell. The chelation of Ca^{++} and Mg^{++} ions could conceivably disturb the integrity of the cell surface, at least insofar as they might be necessary for the activation of cation transport mechanisms. At least one enzyme active at the red cell surface—stroma apyrase—requires either ion for its activity (Herbert, 1956). Tosteson and Robertson (1956) have reported an acceleration of K^+ influx into Duck red cells incubated with ethylenediaminetetraacetic acid in the absence of Mg^{++} and Ca^{++}. We have found, however, that SED in concentrations up to 5·4 μmoles/ml. packed cells, incubated with red cells for up to 60 minutes at 37° C., does not cause a detectable prolytic K^+ efflux.

Some haemolysis was noted, being greatest (3 p.c. cells lyzed) after 60 minutes incubation; this was wholly responsible for the K^+ found in the supernatant. This haemolysis, which was greater than any observed after 60 minutes incubation with Pb (0-0·2 p.c. cells lyzed), did not occur if Pb was present before adding SED.

SED and Lead. The effects of adding SED to red cells before Pb, and the mixture of SED and Pb before the exposure of cells to the metal, were studied.

In the latter instance, there was no significant loss of K^+ into the supernatant within 60 minutes, even if the metal and SED were mixed only 30 seconds before exposure to the cells. This was found to be the case even with the lowest concentrations (0·2 μmoles/ml. packed cells) of SED studied. On the other hand, pre-incubation of SED (0·2 μmoles/ml. packed cells) with the cells was followed by a significant loss of K^+ when Pb was subsequently added. This seemed to be directly related to the time that the SED and cells were in contact, before the addition of Pb (Fig. 4).

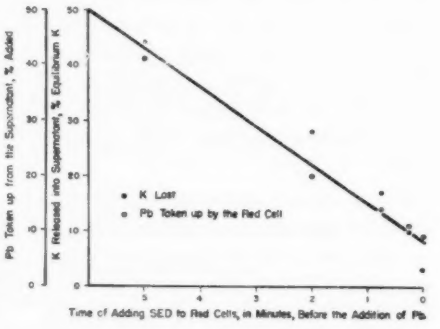

Pb Taken up from the Supernatant, % Added
K Released into Supernatant, % Equilibrium K
K Loss
Pb Taken up by the Red Cell
Time of Adding SED to Red Cells, in Minutes, Before the Addition of Pb

Fig. 4. *The effect of pre-incubation of red cells with SED on the subsequent action of lead.* 0·2 μmoles SED/ml. packed cells was incubated with red cells at 37° C. before the addition of 0·2 μmoles Pb/ml. packed cells. The release of K and the uptake of Pb during the 60 minutes after adding the metal are compared.

Pb was recovered from the supernatant of samples in which it had been mixed with SED before adding cells, to the extent of 91 p.c. This is comparable with the recovery of 90 ± 5 p.c. for the estimation of Pb in the presence of SED alone. Where the SED had been mixed with the cells 5 or 2 minutes before mixing with Pb, the recovery was less than this optimum (Fig. 4), but with a pre-mixing period of less than 1 minute it was almost complete.

These results show that Pb in solution is bound to SED very rapidly under these conditions, and that subsequently, at least within 60 minutes, it is not removed to the red cell to any significant extent, nor does it exert its effect of causing a loss of K^+. However, there is a suggestion that SED pre-incubated with red cells is less efficient in binding Pb, in that a lowered concentration of the metal is found in the supernatant. Under these conditions, a loss of K^+ suggestive of a Pb effect on the cells was observed.

Using a technique similar to that described previously in time studies of the effect of Pb (Vincent, 1958), Pb was added to red cells in the ratio of 0·2 μmoles/ml. packed cells and the mixture incubated at 37° C. Aliquot 5 ml.

samples, equivalent to 1·0 ml. packed cells were removed as incubation proceeded and mixed immediately with 1 ml. of an isotonic solution containing either 0·2, 2·7 or 5·4 μmoles SED/ml. Samples thus prepared were then returned to incubation for the remainder of 60 minutes, when the cells were separated and the supernatant K^+ and Pb were estimated. For each duplicate sample removed, 2 controls were prepared:

(a) one in which a replicate aliquot was added to 1·0 ml. of the suspending medium and incubation completed for 60 minutes;

(b) one, prepared similarly to (a), which was centrifuged immediately and the supernatant removed.

Supernatants from the control tubes were assayed for Pb and K^+ content.

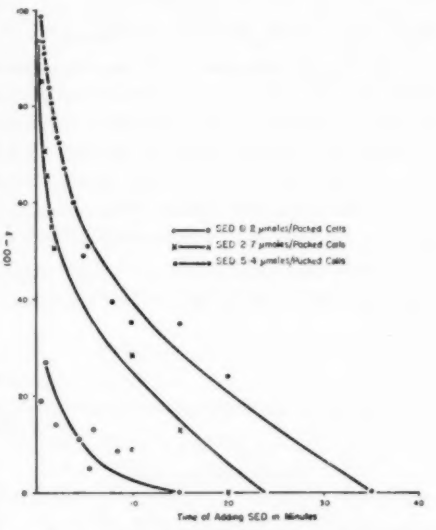


Fig. 5. *The effect of SED, in three concentrations, on the loss of K from red cells previously poisoned with Pb.* The effectiveness of any inhibition is measured by the expression (100-y′) (see text). Pb concentration; 0·2 μmoles/ml. packed cells. Temperature 37° C. Period of incubation: 60 minutes.

A decreased release of K^+ from red cells which were treated with SED, subsequent to Pb, is shown in Fig. 5. If Pb is added to red cells at O minutes, SED added at t minutes, and incubation completed at 60 minutes, the difference between the K^+ concentration in the supernatant at 60 minutes (y) and that present at t minutes (k) represents the loss of K^+ subsequent to the introduction of SED. The loss over the same period – t minutes to 60 minutes – in the absence of SED is equal to (e-k), where e is the K^+ found in the supernatant when equilibrium is attained at 50 minutes. The differences between the loss subsequent to time t in the absence of SED, (e-k), and that in the presence of SED, (y-k)—i.e. (e-y)—is a direct measure of the inhibitory effect of SED. If all the K values are expressed as a percentage of the equilibrium value of 60 p.c., the expression (e-y) becomes (100-y′), where $y' = y/e \times 100$. It is plotted (Fig. 5) in this form against the time of adding SED, where the relative effectiveness of 5·4, 2·7 and 0·2 μmoles SED/ml. packed cells is shown.

The Pb bound to SED, and thus recoverable in the supernatant, was only determined for concentrations of 0·2 μmoles Pb/ml. packed cells. No significant variation with time was observed, the standard deviation of the mean of all values being less than the experimental error of the method of estimation. The

mean Pb recovered was 80 ± 4 p.c. of that added, compared with a recovery of 90 ± 5 p.c. from solutions of SED and Pb alone.

If this difference of 10 p.c. is significant, it must be accounted for by Pb bound to the red cell. It could not be the reason for the relative ineffectiveness of 0·2 μmoles SED, however, because Pb in the concentration of 0·02 μmoles/ml. packed cells is virtually without effect on K^+ release. It would seem that, although a large proportion of the Pb which had been bound to the red cells was removed by an equimolar concentration of SED, the reduction in the loss of K^+ from such cells was relatively slight.

The possibility that Ca^{++} ions may compete with Pb^{++} ions for SED has been investigated briefly. The evidence suggests that if Ca is present before

TABLE 3.

The effects on K release from Pb-poisoned red cells of the addition of Ca before and after the addition of SED

Time of adding (mins.)		K released at 60 minutes, as p.c. available	
SED	*Ca*		
—	—	54·4 54·4 54·4	54·4
6	4	45·7 47·9 47·9	47·0
6	9	37·0 37·0 35·0	36·5
6	17	35·0 39·0 41·4	38·4
6	—	37·0 37·0 37·0	37·0
—	12	56·5 61·1 51·0	56·2
— no Pb	— 0	0·1 0·1 0·1	0·1
— no Pb	—	0·1 0·2 0·1	0·1

Pb: 0·2 μmoles/ml packed cells, added at t = 0 minutes.
SED: 0·2 μmoles/ml packed cells.
Ca: 1·0 μmoles/ml packed cells.

adding SED to the red cell, the inhibitory effect of the latter is partly curtailed. Its addition after SED, however, is without effect on the inhibition of K^+ loss. The results of an experiment in which an excess of Ca (1·0 μmoles/ml. packed cells) was added, before and after SED (0·2 μmoles/ml. packed cells), to cells which had been treated with 0·2 μmoles Pb/ml. packed cells, is shown in Table 3. Ca by itself is without effect on the K^+ of the red cell under these conditions, and fails to protect against the effects of Pb.

SED and mercury. Preliminary studies of the effects of equimolar concentrations of SED on Hg-binding to the red cell and the subsequent K efflux have been carried out and an inhibition, much less than with Pb, has been observed. Furthermore, the pattern of this effect is quite different from that for Pb.

Introduction of equimolar SED into a red cell-Hg system which had been incubating for longer than 0·5 minutes was completely without effect on the K^+ lost from the cells by the end of 60 minutes. It will be remembered that similar treatment of a Pb/red cell system before 10 minutes' incubation had elapsed resulted in a partial inhibition of K^+ loss. The difference becomes more striking when the figures for the uptake of metal by the cells are compared. SED removed Pb from the red cell, even after 30 minutes' incubation,

TABLE 4.

Studies on the inhibition of Hg effects on the red cell by SED
0·8 μ moles Hg/packed cells was added at 0 minutes, 0·8 μ moles SED/packed cells at the times shown, and incubation completed for 60 minutes at 37° C.

Time of adding SED (minutes)	Supernatant K, p.c. available		Hg removed from supernatant, μ moles/ml packed cells	
−10·0	36·4		·43	
	33·8	35·1	·42	·42
−5·0	22·6		·30	
	25·9	24·2	·42	·36
−2·0	22·2		·43	
	26·8	24·5	·41	·42
−0·5	24·7		·39	
	26·8	25·7	·37	·38
pre-mixed 5 minutes	16·3		·35	
	15·1	15·7	·33	·34
0·5	38·1		·48	
	39·6	38·5	·50	·49
1 to 30 minutes means of 16 readings	81·5±2		·72±·02	
SED not added	79·5		·71	
	81·0		·71	
	82·3		·71	
	82·3		·72	
Mean	81·2±1·5		·71±·01	

to the extent of 80 p.c. of the added metal, whereas it completely failed to remove any Hg if added later than 0·5 minutes after incubation had commenced. The mean of 18 readings of supernatant Hg in samples to which SED was added later than this critical time was identical (9·4 ± 0·2 p.c.) with that found in the absence of SED (see Vincent, 1958). As with Pb, the technique of estimation was satisfactory in the detection of Hg in the presence of SED, 91 ± 1 p.c. of added metal being recovered in controls.

If Hg was pre-incubated with SED in equal proportions for 5 minutes prior to its addition to cells, only 16 p.c. of the available cell K^+ was subsequently lost in 60 minutes. If, however, the SED was pre-incubated with the cells before the addition of Hg, the subsequent loss of K^+ was greater—between 25 and 35 p.c. The results of all these experiments are set out in Table 4, where it will be seen there is a suggestion that the longer SED and cells are in contact before the addition of Hg, the less inhibitory is the effect of SED. When the loss of K^+ from cells treated with Hg and SED is compared with the amount of Hg removed from the supernatant, the relationship shown in Fig. 6 is obtained. This corresponds to the relationship between K^+ loss and the concentration of Hg added in the absence of SED, as shown by the open circles, and is the same whether the SED is added before, with, or after Hg. It would seem, then, that K^+ lost from Hg-treated cells to which SED is added is quantitatively related to the amount of Hg retained by the cells in the presence of SED.

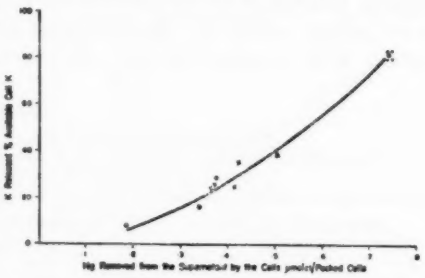


Fig. 6. *The release of K from the red cell after 60 minutes compared with the uptake of Hg in the same time.* The effectiveness of adding equimolar SED to red cells, treated with 0·8 μmoles Hg/ml. packed cells at different times, is shown.

- ● SED and Hg mixed before adding to red cells.
- × SED and red cells mixed before adding Hg.
- ▲ SED added to red cells 30 seconds after Hg.
- • SED added to red cells later than 30 seconds after Hg.

It will be seen that in every instance the K lost can be related to the Hg removed from the supernatant, and that this relationship is the same as that obtained by the use of different concentrations of Hg in the absence of SED (points O).

Simple red cell fractions. Stromata, lipid-free stromatin and haemoglobin were studied in competition with red cells for the uptake of Pb, this being measured by the degree of K^+ loss after incubation. The fractions were each prepared in separate centrifuge tubes from equal volumes of the cells which were subsequently studied.

Pb, in concentrations of from 0·1 to 0·01 μmoles/ml. packed cells, was added to 1 ml. packed cells plus stromata derived from 1 ml. packed cells, and incubated for 30 minutes.

Corresponding preparations without stromata were incubated at the same time, and the loss of K^+ from the red cell is related to the Pb added by the curve shown in Fig. 7. It will be seen by comparison that the loss from red cells in the presence of equivalent stroma is roughly half that expected for the same concentration of Pb, suggesting that Pb is partitioned equally between the stroma and the red cells.

Lipid-free stromatin and haemoglobin, investigated by the same method, were found to be without effect on the loss of K^+ from the red cell.

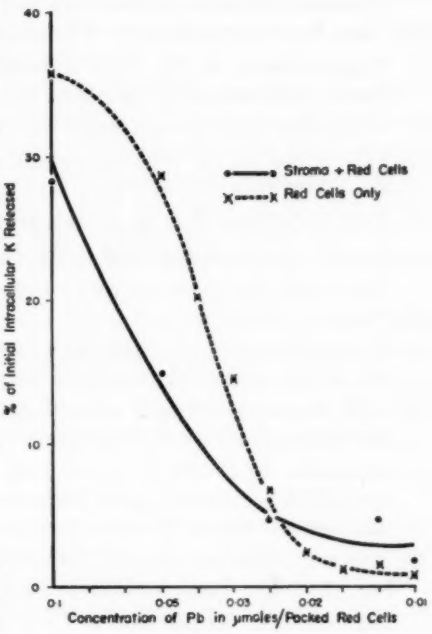


Fig. 7. *The effect of an equivalent quantity of stroma on the Pb-induced loss of K from red cells.* Different concentrations of Pb were incubated for 60 minutes at 37° C.:

(a) with red cells alone;

(b) with red cells plus an equivalent quantity of fresh stroma.

The inhibitory effect of the stroma can be seen to reduce the K efflux to that equivalent to half the Pb concentration.

DISCUSSION.

The results set out above show that Hg and Pb differ, not only in their effects on the red cell, but also in their reaction to different inhibitors. No significant inhibition of the effects of Pb on the red cell by thiosorbitol or cysteine was found in concentrations up to 50 times those of Pb. Passow and Schutt (1956) found that cysteine slightly reduced K^+ loss if used in concentrations 200-250 times those of Pb.

Two moles of thiosorbitol bind one mole of Hg, and mixtures of the two in this ratio have no effect on the red cell. Ratios less than this have an effect equivalent to the unbound Hg, this relationship holding true down to a minimum ratio which is greater in the presence of higher concentrations of Hg. With cysteine, however, the red cell seems able to compete for Hg, for the effects of mixtures are greater than those due to unbound Hg alone.

This difference would be the more interesting if the affinity constants between mercury and thiosorbitol, and mercury and cysteine, were known; unfortunately no reference to such data could be found in the literature.

Clear-cut differences between Pb and Hg with respect to their inhibition by SED have been demonstrated. The results for Hg may be summarised:

(a) Pre-incubation of Hg with equimolar concentrations of SED results in partial inhibition of K^+ loss from the red cells;

(b) Equimolar SED pre-incubated with red cells is less inhibitory than (a);

(c) SED added to red cells 30 seconds after Hg partly inhibits K^+ loss, to a less extent than (a) or (b);

(d) SED added later than 30 seconds after Hg failed to influence the loss of K^+;

(e) Any K^+ lost is proportional to the Hg not found in the supernatant.

The results for Pb mixed with equimolar or excessive (14 or 28 x Pb) concentrations of SED are:

(a) Pre-incubation of Pb with SED completely inhibits its effects on the red cell, all the added Pb remaining bound to the SED;

(b) SED pre-incubated with red cells is less inhibitory than in (a), the reduction being proportional to the period of pre-incubation;

(c) Equimolar SED added to red cells after their exposure to Pb removes roughly 90 p.c. of the metal from combination with them, but exerts little effect on the loss of K^+ subsequent to its addition;

(d) Excessive concentrations of SED added after Pb are more inhibitory to K^+ loss, the effect being related to SED concentrations.

Differences in the partition of Pb or Hg between SED and the red cell are well marked. Red cells can take up 56 p.c. of the Hg present in solution with an equimolar concentration of SED, but none of the Pb; and the same concentration will only take up any Hg in the presence of red cells if added within 30 seconds of the metal. On the other hand, equimolar SED will take up almost all of the Pb bound to the red cell.

The loss of K^+ from cells treated with SED and Hg is equivalent to the effects of the metal bound by the red cell, but in the case of SED and Pb it is far greater. Thus, although equimolar concentrations of SED will remove most of the Pb from combination with the red cell in 24 minutes, the loss of K^+ subsequent to its addition is almost as great as that which would be expected without it. That this post-SED loss of K^+ is less if higher concentrations of SED are used suggests that the rate of removal of the metal is the critical factor determining the effectiveness of the inhibition. A quantitatively greater binding of the metal at the same rate could only account for the removal of another 0·02 μmoles of the added Pb, a reduction in concentration which has little effect on the loss of K^+. If the effects of higher concentrations of SED are due to a more rapid removal of bound Pb the continued presence of the metal on the red cell must be essential to its action. Were its effects irreversible, no concentration of SED would be expected to inhibit them, since equimolar SED can remove the Pb to such a large extent.

These results are in general agreement with those of Passow and Schutt (1956) who concluded that Pb is not bound irreversibly to the red cell, and suggested that it can be transferred from cell to cell.

SED pre-incubated with red cells was less effective in preventing the effects of both Pb and Hg, the greater K^+ loss in both cases being proportional to an increased binding of the metal to the cell. This suggested that the decreased affinity between SED and the metal might be due to a chelation resulting, for instance, from the removal of a cation to the SED in solution or the association of SED with the cell through a cation link. Preliminary studies with Ca^{++} show that this may be the type of cation concerned, SED being less inhibitory if first incubated with it.

Although the affinity constant between SED and Pb is known ($pK_a = 18{\cdot}2$; Martell and Calvin, 1952), there is no corresponding figure for Hg in the literature. The differences recorded here could indicate that the red cell/Hg affinity is greater and/or that the SED/Hg affinity is less, than is the case for Pb. Not until the affinity between SED and Hg is calculated will a more accurate assessment be possible.

Since it competes equally with red cells for the uptake of Pb, red cell stroma itself seems to bind Pb as well as the whole cell. This suggests that the site of binding of Pb is at the cell surface, and the failure of lipid-free preparations to compete suggests that the lipid constituents of the membrane may play a part. The possibility that Pb could associate with the red cell either by binding to a hypothetical lipid-soluble, lipid-non dissociable K^+ carrier (Solomon, 1952), or by solution in the lipid phase in which it functions, has been discussed previously (Vincent, 1958). Either site for Pb binding could explain its inhibition of K^+ transport.

A consideration of the order of effectiveness of the several metals studied ($Pb^{++} > Hg^{++} > Au^{+++} > Cu^{++} > Cd^{++} > Zn^{++}$ – see Vincent and Blackburn, 1958) sheds little light on the general problem of receptor sites for their adsorption to the red cell surface. The order of effectiveness bears no relationship to any of the affinity series for the interaction of metals with proteins (amino acids, pteridines, sulphhydryl or hydroxyl groups–Klotz, 1954). Since the observed differences cannot wholly depend on binding to any of these sites it can be suggested either that the metals bind to different sites or that their activity is not related to their affinity for cell components. It is possible that the far greater activity of Hg and Pb is due to specific high-affinity binding–for instance, to – SH or even – OH groups in the case of Hg, and possibly to lipid in the case of Pb. In this case, the order of effectiveness of the remaining metals is similar to the affinities quoted above, so that differences in binding for any site could explain the greater activity of Cu than Cd and Zn. The unlikelihood that all the metals are bound to SH groups has been shown previously (Vincent and Blackburn, 1958).

Acknowledgments. The author wishes to thank Professor C. R. B. Blackburn for his help and advice during the course of this work, and especially for his constructive criticism of the material presented here.

Thanks are also due to Mr. F. R. Barrett and Mr. A. W. Findlay, of the School of Public Health and Tropical Medicine, for their painstaking estimations of mercury and lead respectively, to Miss J. W. Gray for her careful preparation of the manuscript, and to the Department of Illustration in the University of Sydney for the drafting of the figures.

REFERENCES.

Aub, J. C., Fairhall, L. T., Minot, A. S., and Reznikoff, P. (1925): Medicine, 4, p. 1.
Ballantine, R. (1944): J. cell. comp. Physiol., 23, p. 21.
Drabkin, D. L. (1949): J. biol. Chem., 164, p. 703.
Herbert, E. (1956): J. cell. comp. Physiol., 47, p. 11.
Jandl, J. H., and Simmons, R. L. (1957): Brit. J. Haemat., 3, p. 19.
Klotz, I. M. (1954): A Symposium on the Mechanism of Enzyme Action. W. D. McElroy and B. Glass eds. p. 257. Johns Hopkins Press, Baltimore.
Martell, A. E., and Calvin, M. (1952): Chemistry of the Metal Chelate Compounds. Prentice-Hall, N.J.
Passow, H., and Schutt, L. (1956): Pfluger's Archiv f.d.g. Physiol., 262, p. 193.
Prankerd, T. A. J., and Altman, K. I. (1954): Biochem. J., 58, p. 622.
Solomon, A. K. (1952): J. gen. Physiol., 36, p. 57.
Tosteson, D. C., and Robertson, J. S. (1956): J. cell. comp. Physiol., 47, p. 147.
Vincent, P. C. (1958): Austral J. exp. Biol., 36, p. 589.
Vincent, P. C., and Blackburn, C. R. B. (1958): *Ibid.*, 36, p. 471.

THE INFLUENCE OF CENTRIFUGATIONAL TREATMENT UPON HAEMATOCRIT VALUES AND THE TRAPPED PLASMA CORRECTION FACTOR OF SHEEP BLOOD

by V. ELIZABETH HODGETTS

(From the Division of Animal Health and Production, C.S.I.R.O., Animal Health Research Laboratory, Parkville, Victoria).

(*Accepted for publication 31st October, 1958.*)

SUMMARY.

The influence of the intensity of centrifugational treatment upon the magnitude and precision of haematocrit values of sheep blood, and upon the proportion of trapped plasma, was investigated.

Examination of the concept of Dole and Cotzias (1951), which implies that equivalent multiplication products of RCF and time (i.e., impulse) have the same centrifugational effects failed to support it insofar as sheep blood is concerned; for this the relationship between impulse and haematocrit is represented by a family of hyperboloid curves each corresponding to a particular value of RCF. In the determination of haematocrit values with sheep blood, more intense centrifugational treatment is desirable than has been found adequate for human blood.

The recommended centrifugational conditions when the radius of gyration measured to the bottom of the tube is 17 cm. are 5000 rpm. ($\equiv$ 4756 *g.*) for 60 min. Under these conditions the variance is low, differences in haematocrits of 0·17-0·20 between the means of duplicates being significant ($P = 0{\cdot}05$) in the range 20-40, and over the range 24-53 the trapped plasma correction factor is approximately 0·97, being related to haematocrit by the linear equation, $y = 2{\cdot}26 + 0{\cdot}03x$, where y = p.c. trapped plasma and x = haematocrit value.

INTRODUCTION.

Techniques for determining relative red cell volume in blood by the haematocrit method have been developed mainly for application to human blood (Gregersen, 1951; Reeve, 1952). For this purpose it has been found convenient and adequate to centrifuge blood for 30 minutes at a relative centrifugal force (RCF)[1] of approximately 1500 g. Complete packing of human red cells does not occur under these conditions (Millar, 1925). The "trapped plasma correction factor" (TPCF) for estimating the "true" relative cell volume from the observed haematocrit of human blood has been determined under the conventional centrifugational conditions by several authors, the most reliable estimates being between 0·97 and 0·95 (Gregersen, 1951). This factor does not neces-

[1] $RCF = 1{\cdot}119 \cdot R^2 \cdot r \cdot 10^{-5}$, where R = revolutions per minute (rpm) and r = radius of gyration in cm.; by convention, r is measured to the bottom of the tube.

sarily apply to the blood of other species; indeed, it has been known for 20 years that sheep blood does not "pack" as readily as human blood (Kennedy and Millikan, 1938). Nevertheless, some authors have adopted techniques and data which are strictly applicable only to human blood.

Experiments were, therefore, carried out with sheep blood to determine the influence of the intensity of centrifugational treatment (i.e., gravitational field and time) upon the magnitude and precision of its haematocrit value, and to establish the TPCF under defined conditions.

Methods.

Centrifugation. Since the liquid tachometer could not be read to closer than 50 rpm the RCF, which is derived from the second power of R, is subject to errors of about ± 2 p.c. at 5000 rpm and $\pm 3{\cdot}4$ p.c. at 3000 rpm. The times were measured from when the required speed was reached to when the motor was switched off.

Haematocrit values. Heparinized blood was centrifuged in calibrated Wintrobe tubes of internal diameter 3 mm., the radius of gyration at the bottom of the tube being 17 cm. The tubes were filled not to the 100 mm. mark, but randomly to any point between 90 and 100 mm. They were then sealed by a piece of "Cellophane" tape. After centrifugation, the total height and the height of the cell deposit, including the buffy coat, were estimated to the nearest one-tenth of a mm.; from these values the percentage of cells was calculated. Subsequent analysis of the records of over 2000 readings revealed an unsuspected tendency to favour certain integers (see Mainland, 1938), but, fortunately, this was found to have very little effect upon the means.

Estimation of TPCF. The determination of TP was based upon the "direct" method of Leeson and Reeve (1951), involving the use of ^{131}I-labelled serum. Samples of labelled homologous serum were prepared for each animal. These were stored in a refrigerator, and were used within three days. After the addition of 1 ml. of ^{131}I-labelled serum to 10 ml. of heparinized blood, four Wintrobe tubes were filled with each blood sample, in order to provide sufficient duplicate material for counting. The haematocrit was determined by centrifuging at 5000 rpm (4756 *g.*) for 60 min. The plasma was drawn off very carefully, and as thoroughly as possible, from above the packed cell columns. Residual plasma was then removed by carefully washing the inside of the tubes above the cell columns with saline citrate solution in bulb pipettes until the counts of the washing fluid were not significantly greater than the background counts. The resulting cells from two Wintrobe tubes were then pooled, and suspended in a standard volume of diluent. After removal of cells by centrifugation, the activity of the resulting solution was determined in a liquid-type counter. By comparison with the activity of the supernatant plasma, the percentage of TP and the correction factor were determined. From this value for TPCF the "true" or corrected haematocrit value was estimated and by determining the haematocrit values at 3000 rpm after 30 min. and 60 min., the TPCF under those conditions was calculated.

EXPERIMENTAL.

Precision of haematocrit as influenced by intensity of centrifugational treatment.

The influence of intensity of centrifugational treatment upon the precision of the haematocrit was examined in three samples of blood. Sets of six haematocrit tubes were used for each series of tests. They were centrifuged at 3000

TABLE 1.

Influence of RCF and of time upon magnitude and precision of haematocrit.

Sample	1712 *g*/30 min. (a)			1712 *g*/60 min. (b)			4756 *g*/30 min. (c)			4756 *g*/60 min. (d)			Ratio of haematocrits (d/a)
	H	Var_s	SD_s	H	Var_s	SD_s	H	Var_s	SD_s	H	Var_s	SD_s	
1	37·2	0·174	0·42	34·5	0·074	0·27	32·6	0·030	0·17	32·4	0·028	0·17	0·87
2	47·2	0·178	0·42	41·6	0·066	0·26	39·7	0·014	0·12	39·3	0·020	0·14	0·83
3	61·3	0·238	0·49	54·2	0·238	0·49	48·2	0·010	0·10	47·5	0·038	0·19	0·77

H = Mean haematocrit (6 determinations)

rpm or 5000 rpm (1712 g. or 4756 g., resp.), and were read after 30 min. and again after 60 min. The results (Table 1) showed that increased RCF and time were followed by increased packing, the haematocrits after 4756 *g.* for 60 min. being only 77-87 p.c. of those determined after 1712 *g.* for 30 min. More important, the haematocrit determinations after 4756 *g.* for 60 min. were considerably more precise than those determined at 1712 *g.* for 30 min., the ratio of the mean variances being 0·146. We have accordingly adopted as a routine centrifugation of sheep blood at 4756 *g.* for 60 min. It was shown, by the "t" test, assuming lognormal distribution of haematocrit values, that under these conditions differences of 0·17-0·20 between the means of duplicates, in the range 20-40, are significant at the 5 p.c. level.

The trapped plasma correction factor.

TP was estimated as described on samples collected on two consecutive days from each of six Merino sheep. Four of these were intact healthy animals, and two were splenectomized animals in apparent good health but with haematocrits on the low side of the normal range. Two of the samples from the intact sheep were used to prepare haemoconcentrated samples. For this purpose, the blood was lightly centrifuged, portion of the supernatant plasma was discarded, and the cells were resuspended in the remainder to provide samples with high haematocrit values. The values for TP, after centrifugation at 1712 *g.* for 30 min. and for 60 min., and at 4756 *g.* for 60 min., are given in Table 2, the values

TABLE 2.

Relationship between intensity of centrifugational treatment and the percentage of trapped plasma in the cell column.

Sample	Trapped plasma (p.c.) after			Haematocrit value (at 4756 *g*/60 min.)
	1712 *g*/30 min. (a)	1712 *g*/60 min. (b)	4756 *g*/60 min. (c)	
1	19·4	12·3	3·8	53·2
2	22·7	13·1	3·8	49·0
3	18·6	10·4	3·3	38·5
4	11·5	5·8	3·0	31·7
5	15·3	8·6	3·2	30·8
6	16·0	10·6	3·2	30·5
7	14·4	7·8	3·1	29·4
8	14·2	7·5	3·2	29·2
9	12·4	7·2	3·3	29·3
10	14·3	8·2	3·3	27·8
11	8·2	5·8	3·0	26·6
12	7·6	4·7	2·7	24·9
13	12·5	7·4	3·0	24·6
14	12·7	7·1	3·1	24·2
Mean	14·27	8·32	3·21	
S.D.	4·10	2·46	0·30	
Var.	16·79	6·05	0·09	

of the means being in the ratio 4·45 : 2·59 : 1, and the ratio of the variances 187 : 67 : 1. Under the recommended conditions (4756 *g.* for 60 min.), the mean value of TP for samples within the usual range of haematocrit (24-32) was 3·10 ± 0·17 p.c., and over the full range (24-53) it was 3·21 ± 0·30 p.c. For most purposes it may therefore be taken as approximately 3 p.c., the TPCF being 0·97.

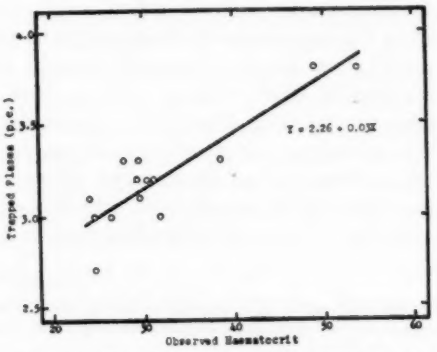


Fig. 1. Regression of trapped plasma on observed haematocrit of sheep blood.

In Fig. 1 the values of TP are plotted against haematocrit. Over the full range of haematocrit values the observations may be fitted to a straight line, the linear regression of TP on haematocrit being TP = 2·26 + 0·03 haematocrit. Within this range, therefore, a more accurate estimate of TP can be made by means of the regression equation.

Relationship between intensity of centrifugational treatment and haematocrit.

The experiments summarized in Table 1 indicate that the value of the observed haematocrit is considerably influenced by the intensity of the centrifugational treatment, both RCF and time contributing to the result. It is therefore of interest to determine their relative importance. Dole and Cotzias (1951) have recommended using the multiplication product of RCF and time to yield a single datum for specifying all centrifugational procedures. This view assumes that identical force-time products, however derived, have the same centrifugational effects. In accordance with the recommendation of Hlad and Holmes (1953), who point out that the force-time multiplication product is a logical extension of the physical variable impulse (I), the product is here expressed in conventional physical units as dyne-sec. $I = \int F \cdot dt$, where F = force and t = time. When F is constant over a given time interval, $\int F \cdot dt = F \cdot t$, and is equivalent to RCF·980·t. Hlad and Holmes investigated the relationship between impulse and the p.c. of trapped plasma in the packed cell column of human blood. Their results show that the Dole-Cotzias concept applied only approximately in this instance, since the relationship was not hyperbolic but hyperboloid. They did not directly investigate the relationship between impulse and haematocrit. If a similar relationship exists, and if its mathematical function were determined, it should be possible to specify a force-time product needed for a required degree of packing, and to calculate the appropriate value of one component, given the other.

Experiments were therefore carried out to determine the relationship between the impulse to which sheep blood was subjected and the observed haematocrit. Four speeds were used, namely, 1500, 3000, 4000 and 5000 rpm, corresponding to RCF = 428 *g.*, 1712 *g.*, 3044 *g.*, and 4756 *g.* respectively, and the times ranged from 15-410 min.; the haematocrit values are the means of four determinations. A recording tachometer would have permitted integration of the accelerative and decelerative phases, but since this was not available, the time involved in acceleration (20-55 sec.) and deceleration (75-90 sec.) was neglected. Values of I therefore tend to be underestimated by amounts ranging

TABLE 3.

Haematocrit values of a sample of sheep blood after various levels of centrifugational treatment.

RPM	Observed haematocrit					
	15 min.	30 min.	60 min.	90 min.	120 min.	410 min.
1500	73·8	53·7	45·3	40·3		33·6
(428 *g.*)	(0·38)	(0·75)	(1·51)	(2·26)		(10·32)
3000	41·4	36·1	33·9	33·5	33·3	
(1712 *g.*)	(1·51)	(3·02)	(6·04)	(9·06)	(12·08)	
4000	36·1	33·9	33·1			
(3044 *g.*)	(2·68)	(5·37)	(10·74)			
5000	34·0	33·4	32·9			
(4756 *g.*)	(4·19)	(8·39)	(16·78)			

Numbers in brackets under "RPM" refer to RCF; and under "observed haematocrit" to values of I, in dyne-sec. x 10^9.

Estimated haematocrit at complete packing = 31·9.

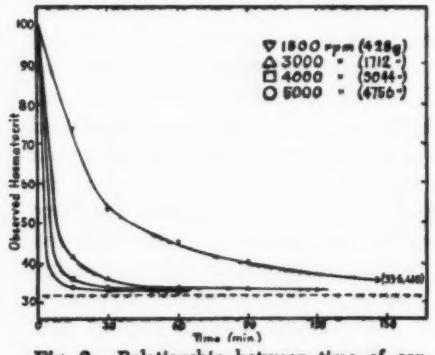


Fig. 2. Relationship between time of centrifugation at various levels of RCF and observed haematocrit. The broken straight line shows the complete-packing haematocrit value.

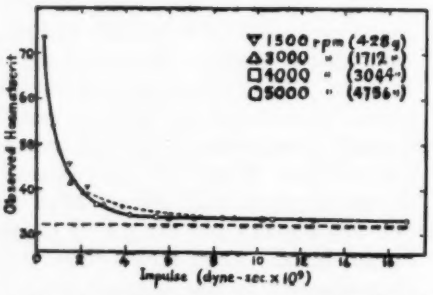


Fig. 3. Relationship between intensity of centrifugational treatment (impulse) of sheep blood and observed haematocrit. The broken straight line shows the complete-packing haematocrit value (31·9). The broken curve indicates the departure of the calculated best-fit hyperbola from the smoothed visual-best-fit hyperboloid curve.

from 0·1 p.c. at low values of RCF and high values of t, to 3 p.c. at high values of RCF and low values of t. By suitable combinations of RCF and time within the practicable range, a considerable amount of over-lapping of values of I was achieved. The data from a typical experiment are shown in Table 3 and the decrease of haematocrit with time for each value of RCF is shown graphically in Fig. 2. The graphs do not make it possible to decide with certainty whether or not the curves would eventually reach a level corresponding to complete packing, namely, 31·9. In Fig. 3, haematocrit is plotted against the calculated values of impulse. On the scale chosen, a visual best-fit line can be drawn to fit the data apparently reasonably well, except for a few points representing RCF = 428 and 1712 g., in the range of $I = 1{\cdot}5\text{-}2{\cdot}3 \times 10^9$ dyne-sec. Plotting of haematocrit against log I and I^{-1} and of log haematocrit against I and log I reveals that the relationship between haematocrit and I is better approximated by a hyperbola than by an exponential or by a power function. The calculated best-fit straight line, when haematocrit is plotted against the reciprocal of impulse, was used to plot the best-fit hyperbola. This departs at the intermediate values from the visual best-fit curve, which may be described as hyperboloid. At this level of discrimination, therefore, the relationship of haematocrit to impulse resembles that reported by Hlad and Holmes between TP and impulse. However, closer inspection of the data on an enlarged scale reveals that this attempt at "smoothing" masks the true relationship, which appears to be represented by a family of curves, probably hyperboloid, each corresponding to a particular value of RCF. Each begins at haematocrit = 100 (i.e., unsedimented blood) and eventually becomes asymptotic to the X-axis (Fig. 3). The data are inadequate to indicate with certainty whether each has its own characteristic asymptote or whether all would eventually reach the same value corresponding to complete packing at haematocrit = 31·9, but the latter appears unlikely.

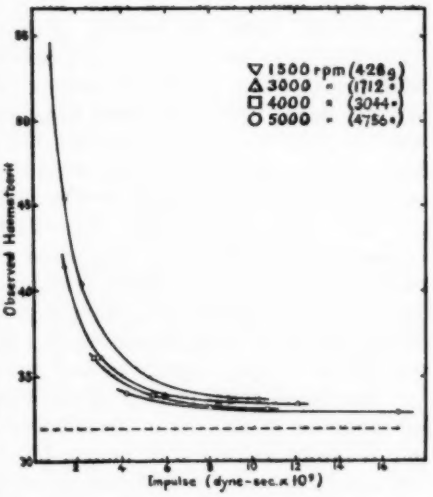


Fig. 4. Relationship between impulse and observed haematocrit. Same data as in Fig. 2, at enlarged ordinate scale, and more critical visual curve fitting, suggesting that the extent of packing is limited by RCF, and that equivalent impulses do not have identical packing effects.

In another experiment with sheep blood, of which the complete-packing haematocrit was estimated as 35·2, haematocrits were determined on samples centrifuged at 5000 rpm (4756 g.) for 30 min., at 1500 rpm

(428 *g.*) for 7 hr., and at 1500 rpm for 23 hr. The impulses at 428 *g.* for 7 hr. and for 23 hr. are 1·26 and 4·14 times the value at 4756 *g.* for 30 min. However, the observed haematocrits at 428 *g.* were slightly but significantly greater than at 4756 *g.* (Table 4). At neither value of *g.* or of I did the haematocrit reach the complete packing value of 35·2, but approached it nearest at the higher value of *g.*

TABLE 4.

Relationship between impulse, at two levels of RCF, and haematocrit.

RPM	RCF (*g*)	Time	Impulse (dyne-sec x 10^9)	Haematocrit
5000	4756	30 min.	8·39	36·9
1500	428	7 hours	10·57	38·2
1500	428	23 hours	34·73	37·2

Estimated haematocrit at complete packing = 35·2.

DISCUSSION.

It is difficult to compare our findings with those of others who have examined sheep blood, because usually the conditions under which the estimations were made have not been sufficiently defined. Most authors have not recorded the radius of gyration of the centrifuge used (Ponder and Saslow, 1930; Whitlock, 1947; Becker and Smith, 1950; Todd *et al.*, 1952; Schambye, 1952; Bunce, 1954) and in one instance the author has only recorded the duration (Holman, 1944). In most instances the level of centrifugation was low, and it is clear from our results that the degree of packing must have been relatively poor, and the precision of the results correspondingly low. This would be especially so in the work of Hansard *et al.* (1953), who applied unusually low RCF (906 *g.* for 30 min., equivalent to $I = 1{\cdot}60 \times 10^9$ dyne-sec.), and yet adopted without further investigation the TPCF determined by Leeson and Reeve (1951) for human blood spun at 1500 *g.* for 30 min., namely, 0·95; from the data presented here it is likely that the degree of packing under those conditions was of the order of only 75 p.c., instead of the assumed 95 p.c. Kennedy and Millikan (1938) clearly defined their conditions.

The Dole-Cotzias concept of the identical centrifugational effects of equivalent impulses is opposed to the observations of Millar (1925), Ponder (1944), and Wintrobe (1948) with human blood and of ours with sheep blood. Our own results are not extensive enough to exclude completely the possibility that, given sufficient time, haematocrits at various levels of RCF might eventually reach the complete-packing theoretical minimum, but it is at least clear that equivalent impulses do not have identical centrifugational effects upon blood and that therefore it is necessary to specify both RCF and time. In this connection it is interesting to note that whereas Kennedy and Millikan (1938) using

18,780 *g.* for 25 min., equivalent to $2{\cdot}76 \times 10^{10}$ dyne-sec., claimed to have obtained complete packing of sheep blood, we obtained only 95 p.c. packing at $3{\cdot}473 \times 10^{10}$ dyne-sec., i.e., with an impulse 26 p.c. greater, obtained by centrifuging at 428 *g.* for 23 hr. The failure of blood to conform to the Dole-Cotzias concept is perhaps not unexpected in view of its complex structure and the progressive changes which occur in it after it is shed; for example, the occurrence of rouleaux formation would introduce more-readily-sedimented aggregates, with different packing characteristics. The temperature generated by prolonged centrifugation might also play a part.

The data in this communication show that for sheep blood the p.c. of TP retained in the cell column is a function of the haematocrit value and that over the range of haematocrits studied the relationship can be adequately described by a linear equation. The dependence of TP upon the magnitude of the haematocrit follows since RCF $\propto$ r. The cells at the top of the column are therefore subjected to a calculably smaller value of RCF than those at the bottom, and the greater the haematocrit, the greater will be the disparity. The situation is undoubtedly more complex than this treatment suggests: it was shown by Hlad and Holmes (1953) with human blood that the distribution of TP in the cell column varied non-linearly from a minimum tending to approach zero at the bottom, to a maximum tending to approach 100 p.c. at the top. The range of variation was greatest at low impulses and least at the upper practical limits of centrifugation. Essentially similar findings were reported by Owen and Power (1953).

It is clear that with sheep blood more intense centrifugational treatment is desirable than has been found adequate for human blood. The conditions adopted by us (5000 rpm for 60 min., when the radius of gyration is 17 cm.) give approximately the same degree of packing as occurs with human blood at 3000 rpm for 30 min., the TPCF being of the same order (0·97), and the haematocrit values are more precise than under less intense conditions.

Acknowledgments. I am happy to express my indebtedness to Dr. A. W. Turner for helpful advice and criticism during the preparation of the manuscript, to Mr. N. E. Southern, Dip.Lab.Techn. (Melb.), for skilled assistance, to Mr. A. M. Downes for instruction in use of counting equipment, and to Dr. E. Binet for valuable statistical help and discussions.

REFERENCES.

Becker, D. E., and Smith, S. E. (1950): Corn. Vet., 40, p. 350.
Bunce, Shirley A. (1954): Brit. vet. J., 110, p. 322.
Dole, V. P., and Cotzias, G. C. (1951): Science, 113, p. 552.
Gregersen, M. I. (1951): Ann. Rev. Physiol., 13, p. 397.
Hansard, S. L., Butler, W. O., Comar, C. L., and Hobbs, C. S. (1953): J. Anim. Sci., 12, p. 402.
Hlad, C. J., and Holmes, J. H. (1953): J. appl. Physiol., 5, p. 457.
Holman, H. H. (1944): J. comp. Path., 54, p. 26.
Kennedy, J. A., and Millikan, G. A. (1938): J. Physiol., 93, p. 276.
Leeson, D., and Reeve, E. B. (1951): J. Physiol., 115, p. 129.

Mainland, D. (1938): The Treatment of Clinical and Laboratory Data, Oliver and Boyd, Edinburgh, p. 224.
Millar, W. G. (1925): Quart. J. exp. Physiol., 15, p. 187.
Owen, C. A., Jr., and Power, M. H. (1953): J. appl. Physiol., 5, p. 323.
Ponder, E. (1944): Medical Physics, edited by O. Glasser, Yr.Bk.Pub., Chicago, p. 598.
Ponder, E., and Saslow, G. (1930): J. Physiol., 70, p. 18.
Reeve, E. B. (1952): Brit. med. Bull., No. 2-3, 8, p. 181.
Schambye, P. (1952): Nord. Vet.-Med., 4, p. 929.
Todd, A. C., Wyant, Z. N., Stone, W. M., and Elam, G. W. (1952): Amer. J. vet. Res., 13, p. 74.
Wintrobe, M. M. (1956): Clinical Hematology, Lea and Febiger, Philadelphia, p. 397.

A SIMPLE RESERVOIR BURETTE FOR ALKALINE SOLUTIONS

by HENRY FRANCIS HOLDEN[1]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

(*Accepted for publication 24th October, 1958.*)

SUMMARY.

A simple reservoir burette for alkaline solutions is described.

EXPERIMENTAL.

For purposes such as the preparation of protein-free blood filtrates (e.g. Nelson, 1944) it is convenient to have a small reservoir burette which protects the baryta solution from the atmospheric carbon dioxide. The one described below is simple and cheap (Fig. 1); unless very large volumes of the solution are used it may well replace the more elaborate and expensive ones that can be procured. It can be readily made by anyone with a slight knowledge of glass-blowing. The reservoir is made from an inverted 300 ml. Erlenmeyer flask of "Pyrex" glass. The entrant air is first washed, in the small bottle housed above the reservoir, with sodium hydroxide solution of about the same strength as the baryta. Below the sodium hydroxide and sealing the tip of the capillary inlet is a little mercury. A slight positive pressure is main-

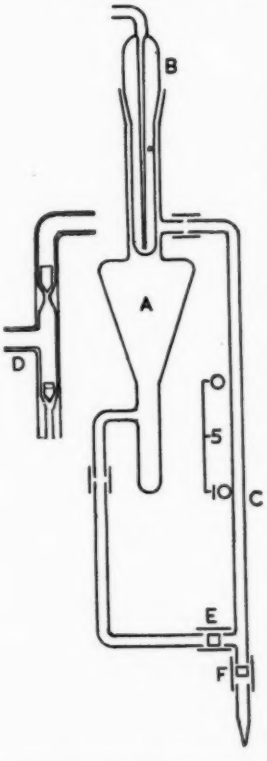


Fig. 1.

A Reservoir with sump for $BaCO_3$.

B Wash-bottle for the entrant air. The capillary ends below the level of the mercury.

C Burette with its zero mark below the level of the outlet from A.

D Air-pump drawn to twice the scale of the reservoir, connected to B with thick-walled rubber tube.

E, F Solid glass cylindrical beads in thin-walled rubber tube to serve as taps for filling and emptying the burette.

The scale is in cm.

[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

tained with the small air-pump shown which is operated with a Pasteur pipette nipple. When not in use the burette outlet is also sealed under mercury in a small tube. The burette consists of a 1 ml. graduated pipette to which were added the extensions shown. Instead of taps, which generally deteriorate too rapidly under the influence of alkalies, small, solid glass beads in rubber tubes are used to control the filling and emptying of the burette. The amount of rubber has been kept as low as possible consistent with reasonable ease in use and maintenance. It is intended that the amount of solution placed in the reservoir shall be used well within the "life" of the rubber and that the latter be replaced when the reservoir is refilled.

REFERENCE.

Nelson, N. (1944): J. biol. Chem., 153, p. 375.